# ACCELERATED AND ENHANCED DIFFERENTIATION OF HUMAN NEURAL STEM CELLS BY A CELL-CONTACT MEDIATED MECHANISM

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**NATIONAL UNIVERSITY OF SINGAPORE**

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Last but most importantly, I thank God for giving me the strength and the intelligence for this pursuit. All who know me well enough would know that this would not be possible without His grace.

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# ABSTRACT

The standard culture methods tend to be inefficient and ineffective in the differentiation of neural stem cells (NSCs) into mature functional neurons. Usually, only a small percentage of NSCs actually differentiate into neuronal cells and the process is generally time consuming, ranging between 1-3 weeks. I hypothesized that the reason for this poor and slow differentiation could be because NSCs do not receive the necessary extrinsic cues for differentiation as they might get in the *in-vivo* system of the intact developing brain. I reasoned that if such cues were provided in a favorable manner to NSCs *in-vitro*, neuronal differentiation could be made more efficient, and more effective. Here, I present a novel co-culture approach that uses a feeder layer consisting of predifferentiated NSCs on which GFP-tagged NSCs are seeded and co-cultured. NSCs normally spontaneously differentiate to various lineages that are at different stages of development; these differentiating cells naturally release soluble factors, deposit their own ECM, exert mechanical stimuli to each other and form a unique nanotopology. As such, a predifferentiating feeder layer of NSCs might replicate some of the natural cues present during brain development.

My results show that NSCs co-cultured on predifferentiated feeder layers undergo accelerated and enhanced neuronal differentiation as compared to cells grown under control conditions. Within 12 hrs of co-culture, GFP-NSCs expressed a neuronal morphology with a marked increase in neurite length and in neurite branching complexity. Furthermore, significant down regulation of NSC markers and notably higher levels of neuronal markers were observed.

Accelerated and enhanced neuronal differentiation could not be achieved on an undifferentiated feeder layer or on predifferentiated NSCs that were fixed using standard fixatives; thus, the feeder layer cells need to be alive and predifferentiated.

In view of understanding the mechanism by which accelerated and enhanced differentiation occurs, I hypothesized that extrinsic factors such as soluble factors, the ECM or direct cell contact could be involved in triggering accelerated and enhanced neuronal differentiation. In the first sets of experiments using condition media or manipulating the concentration of soluble factors within the cell culture medium, I ruled out that soluble factors contributed to enhanced differentiation. I then tested whether ECM plays a role and I observed that the ECM was also not contributing to the accelerated and enhanced neuronal differentiation. Next, I tested whether cell junctions were involved in rapid differentiation. Of the 3 general types of cell junctions (tight junctions, gap junctions and anchoring junctions), tight junctions are generally predominant in epithelial cells and not on neural cells. Thus, I tested the role of gap junctions and anchoring junctions. I found that when gap junctions were inhibited using pharmacological inhibitors, accelerated and enhanced differentiation could not be observed. Furthermore, using blocking antibodies against N-Cadherin in the co-culture conditions abrogated accelerated and enhanced neuronal differentiation.

These data demonstrate a critical role of gap junctions and N-Cadherins (anchoring junctions) in accelerated and enhanced neuronal differentiation of NSCs in the co-culture condition. However, the exact mechanism by which gap junctions and anchoring junctions facilitate accelerated and enhanced differentiation requires further investigation.

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| Symbol | Definition |
|---|---|
| µM / uM | Micro-molar |
| µg | Micrograms |
| α-DDC | Anti-DDC via ICC |
| 18GA | 18 β-Glycyrrhetinic Acid |
| bFGF | Basic FGF |
| CAM | Cell Adhesion Molecule |
| CBX | Carbenoxolone |
| ChAT | Choline Acetyl transferase |
| CM | Conditioned Media |
| CNS | Central Nervous System |
| Control condition | NSCs seeded on laminin |
| DA neurons | Dopaminergic Neurons |
| Day x Feeder | Feeder layer predifferentiated for x days |
| DBH | dopamine-β-hydoxylase |
| DCX | Doublecortin |
| DDC | Dopa Decarboxylase |
| DNA | Deoxyribonucleic Acid |
| DOC | Sodium deoxycholate mediated de-cellularization |
| DOCDOC | Extender DOC procedure |
| DxS / DS | Dextran Sulphate |
| ECM | Extracellular Matrix |
| EGF | Epidermal growth factor |
| eGFP | Enhanced GFP |
| EtOH | Ethanol |
| FACS | Fluorescence-activated cell sorting |
| FGF | Fibroblast growth factors |
| Fig | Figure |
| GAD65 | Glutamate decarboxylase with MW – 65kDa |
| GFAP | Glial Fibrillary Acidic Protein |
| GFP | Green Fluorescent Protein |
| GFP-NSCs | NSCs with GFP protein constitutively tagged NSCs |
| HBSS | Hank's Balanced Salt Solution |
| hESCs | Human Embryonic Stem Cells |
| Hrs | Hours |
| ICC | Immunocytochemistry |
| KCl | Potassium Chloride |
| MAP2 | Microtubule-associated protein 2 |
| MeOH | Methanol |
| Min | Minutes |
| mL | Milliliter |

| Symbol | Definition |
|---|---|
| MMCs | Macromolecular Crowders |
| mRNA | Messenger RNA |
| mV | Milli-Volts |
| N-CAD | N-Cadherin |
| nM | Nano-Molar |
| NGS | Normal Goat Serum |
| NSCs | Neural Stem Cells |
| PA 6 | Bone Marrow-derived Stromal Cell-line |
| pA | Pico Ampere |
| PBS | Phosphate buffered saline |
| PCR | Polymerase Chain Reaction |
| PFA | Paraformeldahyde |
| RE | Restriction Enzyme |
| ReNcell-CX | NSC line derived from the cortex by ReNcell® |
| ReNVM | NSC line derived from the fetal Ventral-Mesensepholon by ReNcell® |
| RNA | Ribonucleic Acid |
| RNAi | RNA Interference |
| siRNA | Small interfering RNA |
| SNARE | SNAP (Soluble NSF Attachment Protein) REceptor") proteins |
| SOX | SOX [SRY (sex determining region Y)-box] family of genes |
| TH | Tyrosine Hydroxlase |
| TPH | Tryptophane Hydroxylase |
| T-SNARE | Target SNARE |
| Tuj1 | Neuron-specific class β-III -Tubulin |
| VGCC | Voltage Gated Calcium Channels |
| VM | Ventral Mesencephalon |
| V-SNARE | Vesicle SNARE |

# CHAPTER 1:

# INTRODUCTION

# Chapter 1: Introduction

## 1.1 The Brain

The study of the brain has been described as the last frontier in biological sciences (Kandel, et al. 2000). It is arguably the most complex organ in an organism and is involved in the life form's exhibition of consciousness. In humans, like in many other organisms, it directs every facet of life (conscious and subconscious) by an intricate network of neurons and glial cells, which interconnect within the brain and extend throughout the entire body. The human brain contains more than a 100 billion ($10^{11}$) neurons of about 2,500-5,000 neuronal cell types (Kandel, et al. 2000). These neurons are distributed throughout the different brain regions and they are aided and sustained by different glial cells. Each neuron has multiple connections to other neuronal types. Though the exact total number of connections is not available, crude approximations show that there are about 100 trillion ($10^{14}$) synaptic connections (Williams and Herrup 1988) forming intricate networks that interconnect with each other. There are numerous discrete pathways which are known to be involved in learning & memory, vision, hearing, cognition, motor coordination, sensory perception, and other essential functions. However, there are many others yet to be understood.

## 1.2 Neurodegenerative Diseases and other Neurological Ailments Disrupts Functional Neural Circuits

Neurodegenerative diseases, neurological disorders and brain injuries deteriorate neurons in the body, thereby disrupting their respective functional pathways. Depending on the respective pathway disrupted, patients suffer a wide spectrum of defects of varied severity. These defects are especially daunting due to the multiplicity of the cell type and

the precision with which they must act both, spatially and temporally within the central nervous system (CNS) (Goldman 2005). For example Parkinson's disease, a neurodegenerative disease that is known to affect 1% of all people above the age of 65, is caused by the degeneration of dopaminergic neurons at the substantia nigra-pars compacta, situated at the basal ganglion. This region is known to different neuronal pathways or circuits that are required in the learning of motor skills, motor coordination, emotions and the execution of voluntary jerky movements at different parts of the body. Some symptoms of Parkinsonism are tremor, rigidity in movement, bradykinesia, lack of coordination, and the impairment of reflexes (Jankovic J 2008). Though drugs and different surgical procedures, including the transplantation of fetal grafts are able to ebb the symptoms, none have led to recovery. Like Parkinson's, other degenerative disorders and injuries pose a threat that has yet to see concrete hope for patients.

### 1.3 NSCs - A Possible Hope?

During development, the entire nervous system originates at the neural plate in a process called neurulation (Bear, et al. 2007). The neural plate folds after 3 weeks of fertilization to create the neural tube and the neural canal. The neuroepithelium, the epithleial cells lining the neural tube rapidly proliferates and precisely subdivides into regionally specific groups that differentiate into distinct neurons and glia that form functional networks that eventually make up the CNS; these cells are thus regarded as the original neural stem cells (NSCs) in the brain (Morest and Silver 2003; Panchision and McKay 2002). As these precursor cells undergo mitotic arrest, they delaminate from the ventricular surface and form a mantle layer that becomes larger and more complex as development proceeds (Panchision and McKay 2002). NSCs are capable of self-renewal

and multipotent differentiation into the three principle cell types of the CNS – neurons, astrocytes and oligodendrocytes (Glaser, et al. 2007). Its differentiation is governed by intrinsic & extrinsic factors (Morrison, et al. 1997; Watt and Hogan 2000; Glaser, et al. 2007). Intrinsic factors include proteins involved in asymmetric cell division, transcription factors controlling gene expression and epigenetic modifications. The extrinsic factors are environmental factors which could be broken down into soluble factors, the ECM, contact with other cells and the general physical environment that may transduce an effect onto the cells (Glaser, et al. 2007; Campos 2005; Emoto 2010; Teo 2010). In conjunction with these intrinsic and extrinsic signals, the cell makes 3 fundamental decisions in order to generate a post-mitotic cell of a particular fate (Altmann and Brivanlou 2001). Firstly, it needs to determine its positional identity within the developing system. Secondly, it needs to decide whether to self-renew or undergo mitotic arrest. Thirdly, it must interpret its mitotic arrest using either intrinsically and / or extrinsically derived information that directs its particular fate. This information continually changes during development and it results in either apoptosis, or the different neural fates. Thus, although cells may share a common regulatory mechanism, evidence suggests that extrinsic cues such as positional identity and temporal identity together with its own internal programme are distinct factors to neural differentiation (Panchision and McKay 2002). Though much has been learnt, the details of the particular mechanisms involved are still extensively being studied (Glaser, et al. 2007; Conti and Cattaneo 2010).

NSCs exists in the adult brain and have the ability to integrate with the developed brain, forming functional neuronal networks (Goldman 2005; Goldman 1998; Gage 2002;

Sanai, et al. 2004; Kirschenbaum, et al. 1994; Roy, et al. 2000), thus suggesting neuroplasticity in the adult brain. As NSCs differentiate to make up the entire CNS and as they are able to integrate into the adult system, could these cells be used therapeutically in CNS repair? In contrast to drug-based therapies in neurodegenerative diseases or injuries, NSCs could replace the missing or damaged cells, thereby restoring function. Unfortunately within the brain, not only is there a general shortage of endogenous NSCs for the purpose of repair and it has been shown that these cells are unable to respond in neurodegenerative ailments and injuries (Goldman 2005; Jandial R, et al. 2008). NSCs thus need to be expanded to the appropriate quantity *in-vitro*, differentiated to the particular stage of development that is crucial for the integration into the system and transplanted into the injured brain utilizing existing transplantation strategies (Kallos, et al. 2003).

Though there exist protocols and bioprocessing facilities that expand NSCs to the relevant numbers, the bottleneck lies in our lack of knowledge to efficiently and effectively differentiate NSC (Kallos, et al. 2003); the standard neural culture method is unable to successfully induce a large percentage NSCs to differentiate to particular neural fate (Gritti Angela, et al. 2001; Pollard, et al. 2006; Schumm, et al. 2003).

### 1.4 *In-vitro* Culture of NSCs

NSCs can be isolated from the fetal brain, the adult mammalian CNS and from embryonic stem cells or induced pluripotent stem cells (Fig. 1) (Glaser, et al. 2007; Conti and Cattaneo 2010). Though cells from these sources have self-renewal and multi-potential differentiation, NSCs isolated from the fetal and the adult brains exhibit properties of astrocytes and radial glial cells (Glaser, et al. 2007; Merkle, et al. 2004;

Götz and Steindler 2003; Götz 2003). NSCs are propagated in the presence of mitogenic factors such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Merkle, et al. 2004; Götz and Steindler 2003). Upon the removal of these mitogenic factors, NSCs differentiate spontaneously, yielding a small percentage of cells that differentiate into the 3 neural lineages. Though most cells exhibit a differentiated morphology, a large population of cells continue to express NSC markers such as Nestin and SOX2. Furthermore, the numbers of cells that express neuronal phenotypic markers are extremely low (Glaser, et al. 2007) and it generally takes NSCs 1-3 weeks to differentiate into neuronal cells (Gritti Angela, et al. 2001). This indicates that the *in-vitro* system, without proper modifications, is inadequate for neuronal differentiation. Apart from basic scientific research, the *in-vitro* differentiation of NSCs is for the purpose of using these cells in the clinic for therapeutic applications (Schumm, et al. 2003; Jandial R, et al. 2008). As such, if NSCs are able to differentiate more rapidly (lesser than 1-3 weeks), they would provide more flexibility as waiting time would be eliminated. Thus, an ideal *in-vitro* system should be able to provide an effective neuronal differentiation, at an accelerated rate.

Looking at the *in-vivo* system - the adult or the developing brain – it can be understood that certain fundamental factors need to be present for neuronal differentiation. Each of these properties has a role that would direct a cell toward a particular fate. Thus, to achieve neuronal differentiation it is reasonable to assume that an understanding of the roles of these features and attempts to replicate them in a cell-culture system is necessary.

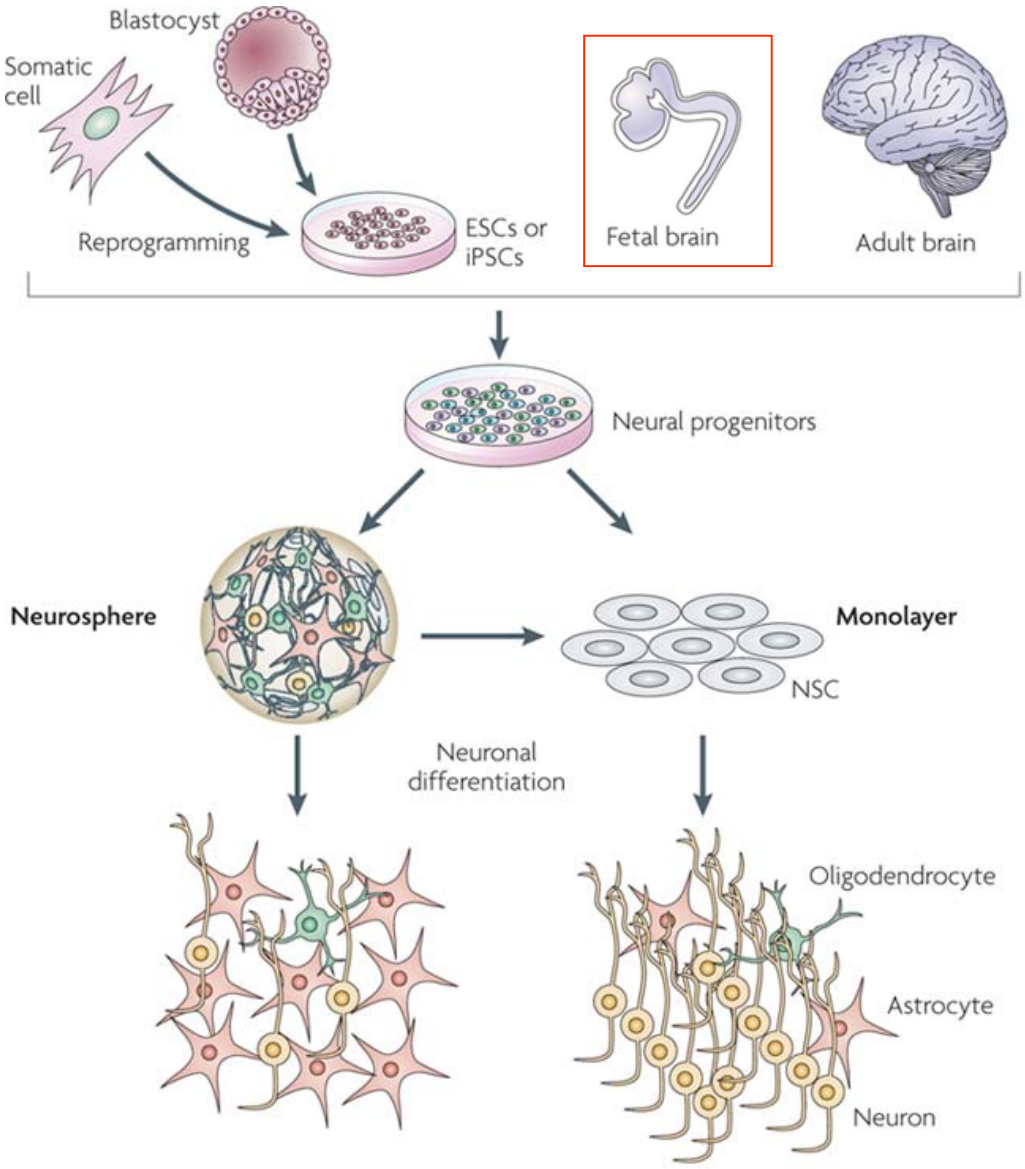


**Figure 1: Derivation of NSCs from their possible sources.** Upon isolation, NSCs could be cultivated as neurospheres or as a monolayer. As seen the image, the differentiation yield for each method is different. The cells used in this project were derived from the fetal brain (red box) and cultured as a monolayer. **(Conti and Cattaneo 2010)**

### 1.4.1 Role of Genetic Modifications

In the literature, the method that has the greatest yield in differentiating NSCs into neurons of a particular fate is by the transfection of Lmx1a (Friling, et al. 2009). Lmx1A is a homeodomain transcription factor that is selectively expressed in proliferating mesencephalic dopaminergic neurons and induced in response to early signaling in the ventral midbrain, where the substantia nigra resides (Ono, et al. 2007; Friling, et al. 2009). To date, this method has produced the largest neuronal yield of dopaminergic neurons. Though this is an excellent proof of principle that the majority population of NSCs may be manipulated to a particular fate, the cells cannot be clinically transplanted into patients due to the risks associated with viral-based vectors involved in genetic manipulation (Marshall 2000). They have also shown to be unreliable in drug screening assays as it has been reported that small transfected molecules affect epigenetic genome modifications and could cause unexpected gene activation, thus giving unreliable screening results (Kaji, et al. 2009).

### 1.4.2 Role of Diffusible Factors

Besides genetic manipulations, extrinsic factors that govern cells during development are used in attempting to direct cells to differentiate to a particular fate; most of these have been concentrated on the extrinsic diffusible soluble growth factors, cytokines, neurotrophic factors and / or other soluble proteins which influence differentiation (Friling, et al. 2009). The known diffusible factors at the localized *in-vivo* region of the brain are supplemented into the cell culture media, so as to coax the cells into differentiating to a particular fate (Hynes, et al. 1995; Hynes and Rosenthal 1999;

Hynes, et al. 1995). The regulation of differentiation, *in-vivo*, involves several transcription factors. These include proneural bHLH factors Ngn2 and Ascl1/Mash1 and their antagonists, the members of the SoxB1 and Hes families (Wegner and Stolt 2005; Holmberg, et al. 2008; Lee 1997; Ross, et al. 2003). However, the exact identity and role of upstream signals controlling these effectors still remain obscure (Gtz and Huttner 2005). Thus, known diffusible factors that mediate these transcription factors have been utilized in directing NSC differentiation. Furthermore, morphogens underlying the regional specification of cells are also utilized in attempt to create the same effect in a culture dish. For example, after the formation of the neural tube, a BMP 4 concentration gradient originating from the roof plate spreads in the ventral direction down to the floor plate. Originating from the floor plate, Sonic hedgehog (Shh) spreads in the dorsal direction also forming a concentration gradient. This double gradient confers a positional identity to the NSCs at the neural tube inducing them towards different mature lineages. Similarly, different FGFs (Altmann and Brivanlou 2001; Mason 2007; Panchision and McKay 2002) and combinations of other diffusible factors are used in a cell culture condition to either keep them in the stem cells phase, undergoing self-renewal (Lahti, et al. 2011), or to direct cells into more mature lineages (Lathia, et al. 2008). However, when used to direct differentiation *in-vitro*, these molecules are less successful. *In-vivo*, these molecules are presented as a concentration gradient to cells of several neuronal cell types that had previously been conferred different regional, positional and developmental cues. As these cues are not present and are difficult to mimick *in-vitro*, they are less successful in directing differentiation as there is limited neuronal yield and efficiency (Friling, et al. 2009).

### 1.4.3 Role of the ECM

Though there has been comparatively lesser studies on the role of the extracellular matrix (ECM) on NSC differentiation, ECM molecules are known to have an impact on neuronal remodeling and directing differentiation (Emoto 2010). In general, 3 types of molecules are abundant in the ECM of any tissue: Proteoglycans, collagen fibers and soluble multiadhesive matrix proteins (Lodish 2008). Proteoglycans are a group of glycoproteins, proteins that contain oligosaccharide chains, which cushion cells and bind them to a spectrum of extracellular molecules. Collagen fibers provide mechanical strength and resilience while the soluble multiadhesive matrix proteins bind and cross-link cell surface adhesion receptors with other ECM components. Thus, the ECM has a dual pronged role - it helps organize cells into tissues, forming and maintaining its 3-dimentional architecture and it coordinates cellular functions by activating intracellular pathways, mediating intercellular communication between cells. Different ECM proteins, and the rigidity of the ECM have been shown to direct the differentiation of NSCs (Du, et al. 2011; Glaser, et al. 2007; Schmid and Anton 2003; Campos 2005). However, as the production and destruction of the ECM is a dynamic process (Benjamin 2001), replicating an *in-vivo* environment in the standard cell culture system is plausible but daunting task.

### 1.4.4 Role of Cell-Cell Contact

Various studies have shown that cell-cell interaction plays a crucial role in the maintenance of the NSC niche (Demir, et al. 2009; Zhang, et al. 2010) and in neural differentiation (Chao, et al. 2009; Temple and Davis 1994; Hatten 1987; Hatten 1985; Gao, et al. 1991; Barakat, et al. 1982). In the stem cell niche, different cell-cell adhesion

molecules and surface antigens provide lateral inhibition to neighboring NSCs, preventing cells from differentiating. A drawback with the standard cell culture system is that NSCs are in contact with each other, which only in approximation reproduces a viable NSC niche.

In the adult as well as the developing brain, as NSCs differentiate, they migrate out of the NSC niche to other regions. During this time, different cells act as guideposts that utilize contact to steer NSCs to their final lineage and destination (Chao, et al. 2009). In 2000, Tsai and McKay (Tsai and McKay 2000) showed that by limiting the cell-cell interaction between stem cells, they changed their morphology and even expressed non-neural specific markers(Gallagher, et al. 2000). Neural cell-cell interaction has also been shown to down-regulate post translational modifications on cell adhesion molecules, such as NCAM, which is involved in neuronal differentiation (Vorasubin, et al. 2007). It has also been observed, that NSCs preferentially differentiate into region specific neurons when they are co-cultured with developing brain slices from respective regions of the brain (McNamara, et al. 2010). Thus, enabling NSCs to have direct interaction with cells of different neural lineages would possibly enhance its *in-vitro* differentiation potential, which is not facilitated in the standard cell culture method.

### 1.4.5 Role of Mechanical and Nanotopographical Cues

In addition to the biochemical signals, and in conjunction with cell-ECM interactions and cell-cell interactions, another dynamic criteria that is difficult to be replicated in the standard cell culture system is the mechanical and nanotopographical control of stem cell differentiation (Teo 2010; Kolahi and Mofrad 2010). Mechanotransduction has been shown to be a major regulator during development and

homeostasis; cells employ various mechanisms, which differ depending upon cell type and environment, to sense and respond to forces (Baharvand 2009). As through development and adult neurogenesis, as NSCs migrate out and differentiate, they interact and respond to different physical stimuli that have been shown to influence stem cell differentiation (Du, et al. 2011; Meyer, et al. 2009; Abrams, et al. 2000). Some of these stimuli include surface texture, elasticity and porosity (McNamara, et al. 2010; Wang and Spector 2009; Meyer, et al. 2009). Attempt to optimize the standard cell culture systems by incorporating these mechanical and nanotopographical features are in process (Meyer, et al. 2009; Wang and Spector 2009; Cheng and Kisaalita 2010; Abrams, et al. 2000; Baharvand 2009; Leipzig and Shoichet 2009; Soen, et al. 2006; Yim, et al. 2007). However, as with the above conditions, the static cell culture system is unable to replicate the dynamic nature of the *in-vivo* system that presents the cells with different physical cues at different stages of development (Ding and Schultz 2004).

To summarize, *in-vivo* neuronal differentiation involves the synergistic interplay of various extrinsic and intrinsic factors, which lead cells to make 3 fundamental decisions that eventually lead to mature functional neuron. Firstly, it needs to determine its positional identity within the developing system. Secondly, it needs to decide whether to self-renew or undergo mitotic arrest and thirdly, it must interpret its mitotic arrest using either intrinsically and / or extrinsically derived information that directs its particular fate. Though it would be impossible to replicate the complexity of the brain in a *in-vitro* system, there is an unanimous consensus for a need to re-engineer the *in-vitro* differentiation culture of NSCs (Glaser, et al. 2007; Meyer, et al. 2009; Teo 2010;

Donato, et al. 2007). I thus attempted to create a cell culture system that contain some fundamental properties or features that drive neuronal differentiation.

### 1.5 Experimental Approach and Rationale of Study

As the ultimate goal is to enable efficient and effective neuronal differentiation that could possibly lead to therapy, our method precludes genetic modifications. We need a system that contains diffusible factors, ECM and a variety of cell types that provide mechanical and nanotopographical cues. NSCs spontaneously differentiate to yield astrocytes, oligodendrocytes, and neurons (Clarke, et al. 2000; Glaser, et al. 2007). It is known that the population of differentiating NSCs *in-vitro* results in a mixture of cells of various lineages and at different stages of development. These cells naturally produce and deposit their own ECM, exert mechanical stimuli to each other and form their own unique nanotopology. They also release diffusible factors; but unlike the *in-vivo* system, these factors are unlikely to form a differentiation gradient.

As a culture of differentiating NSCs contains many of the fundamental properties that are necessary for neuronal differentiation, we created a system that uses this as a platform to differentiate NSCs. I believe my method is more advantageous than co-culture on fetal grafts or on neuronal cells. Fetal grafts are of a restricted brain region and would thus only provide extrinsic cues that are region specific. Neuronal cells, on the other hand, will not provide the spectrum of cells at different stages of development. Thus, the use of predifferentiating NSCs is advantagous as they would provide a complex mix of dynamic signals. In the standard culture system, the outcome of a heterogeneous cell population coming from differentiating stem cells is generally taken to be

undesirable. Here, this ‘undesirable situation is made beneficial because it replicates the variegated interactions present in the developing brain.

NSCs are tagged with GFP and seeded onto a predifferentiating culture of neural cells (originally NSCs). This culture system provides a dynamic biological system that fulfills a larger portion (as compared to the standard culture method) of the fundamental characteristics necessary for neuronal differentiation. Thus, the predifferentiated cells mimic the natural *in-vivo* system so as to propel NSC differentiation.

This thesis then sets out to do the following:

- Characterize the differentiation of NSCs by the conventional standard method
- Implement a novel co-culture method using predifferentiated NSCs as feeder cells.
- Demonstrate that this co-culture system is beneficial in terms of efficiency of neuronal differentiation within a shorter time frame .
- Provide evidence that the differentiated feeder layer is the cause for the observed effects
- Investigate the cellular and molecular mechanisms of accelerated and enhanced differentiation.

# Chapter 2:

# Materials & Methods

# Chapter 2: Materials & Methods

## 2.1 Standard Cell Culture

### 2.1.1 Cell Lines

The ReNcell VM line (ReNVM) was created and distributed by ReNeuron Ltd (Donato, et al. 2007). It was derived from a ten-week old fetal midbrain tissue that was obtained from Kings College Hospital, London. This cell line was used for all experiments, unless otherwise stated.

ReNcell-CX (CX) (Millipore, Billerica, MA) originally derived from the cortex of the fetal brain was also utilized in some experiments, as stated within the thesis.

### 2.1.2 Proliferation

ReNVM cells were cultured on tissue culture ware coated freshly with mouse type 1 laminin (Invitrogen, 20 μg/ml in DMEM:F12) for 1.5 h at 37°C. Cells were expanded for experimental work on laminin coated T75cm2 (BD, Falcon) in growth medium (DMEM:F12 containing B27 neural cell supplement mix (Gibco), heparin (10 Units/ml, Sigma) and Gentamicin (50 μg/ml, Gibco) together with growth factors bFGF (10 ng/ml, Invitrogen) and EGF (20 ng/ml, Sigma). All cells in culture were maintained at 37°C in a humidified atmosphere of 95% air /5% CO2.

### 2.1.3 Differentiation

Cells were passaged onto laminin-coated cell culture ware for differentiation assays. Cells were seeded at 30,000 cells/cm2 and expanded to 90% confluency in growth medium over a 3-4 day period. In general, differentiation was initiated by changing the

medium to that without growth factors. In these experiments, cells were biased toward the neuronal fate by using Neurobasal Media, containing B27 neural cell supplement mix (Gibco), heparin (10 Units/ml, Sigma), Gentamicin (50 μg/ml, Gibco) and L-Glutamine (2mM, Gibco).

### 2.1.4 Sub-Culture

Cells were sub-cultured or passaged every three to four days using trypsin and trypsin inhibitor solutions. Cells were rinsed with HBSS without $Ca^{2+/}Mg^{2+}$ and then incubated in trypsin solution for 3-5 min until the cells detached. Twice the volume of trypsin inhibitor was added and the cells were centrifuged at 1000g x 5min. The cell pellet was resuspended in fresh growth medium and plated in a freshly laminin coated tissue culture ware at a density of ~ 10000 cells/$cm^2$. For consistency, all experiments presented in this study were carried out on cells between passages 25 and 32.

### 2.1.5 Cryopreservation of cell line

Cells were frozen at -80°C and then transferred to liquid nitrogen for long term storage. Cells were frozen in cryovials at a density of 3-6 x $10^6$ cells per ml of cryopreservation media (growth media with 10% DMSO).

## 2.2 Co-Culture

### 2.2.1 GFP tagged NSCs used in Co-Culture

Transfected cells were a gift from Dr. Hui Ting from IMB. In brief, ReNVM cells of passage 23 were transfected with the PT3027-5 eGFP plasmid (Clontech Laboratories, Inc). Transfected cells were sorted at the single cell level and seeded as a suspension in

growth media, for clonal expansion as neurospheres. Positively transfected clones were further expanded and cultivated.

In the co-culture experiments, 10,000 cells / $cm^2$ were seeded onto the predifferentiated feeder layer of cells. The same numbers of cells were also seeded in the control condion.

### 2.2.2 Feeder Layer

NSCs are seeded on the respective cell culture ware coated with Natural Mouse Laminin (20µg/mL) (850kDa) (Invitrogen, USA). Upon reaching 90% confluency, cells were induced to spontaneously differentiate by changing the media to differentiation media. GFP-NSCs were co-cultured with the feeder layer of pre- differentiated neural cells and GFP cells are analyzed between 12 hrs and 7 days of co-culture.

### 2.2.3 Analysis of GFP Cells

Random frames of GFP cels were counted both in the co-culture condition and in the control condition. The morphology of the GFP-NSCs co-cultured on the feeder layer were were quantified by the neurite extensions and the branching complexity. An outlining circle was drawn around the DAPI staining of the nucleus. The area around of the circle was doubled and this was used as the boundry from which measurements were made to the tips of the neurite extensions. The sum of these extensions was taken as the neurite length. As for the branching complexity, the sum of branching points was used as an indicator.

For data analyzed by looking at the expression of specific markers, positive cell counts were normalized with the total number of GFP cells.

### 2.2.4 Creation of ECM

The ECM was created at the Tissue Modulation Laboratory (NUS) under the guidance of A/P Michael Raghunath. The protocols used were as published by Peng et. al. 2011 (in review) (Peng, et al. 2011). ReNVM cells between passages 28-32 were seeded at 30,000 cells/$cm^2$ on 48 well culture plates (BD- Primeria) under standard proliferating conditions in DMEM-F12 (Gibco, Invitrogen, USA). On reaching 80% confluency, culture medium was switched to differentiation media with or without 100μg/ml dextran sulfate 500 kDa (US Biologicals, USA) or 37.5mg/ml Ficoll 70kDa and 25mg/ml Ficoll 400kDa (GE Healthcare, USA). Dextran sulfate and Ficoll were utilized as macromolecular crowders. After 7 days of predifferentiation in the presence of the crowders, cells were lysed with a DOC or DOCDOC lysis protocol. The DOC lysis protocol consists of 3 x 10 minutes incubation on ice with 0.5% sodium deoxycholate (Prodotti Chimici E Alimentari S.P.A., Italy) and 0.5X Complete Protease Inhibitor (Roche, USA). DOCDOC protocol consists of 6 x 10 minutes incubation on ice of 0.5% sodium deoxycholate and 0.5X Complete Protease Inhibitor.

### 2.2.5 Concentration of Condition Media

The conditioned media (CM) was concentrated using a Amicon Ultra-15 Centrifugal Filter Device (Millipore), with a 3000 Nominal Molecular Weight Limit. The concentration of the CM was quantified based on volume. The concentrated CM preparations were filtered with a 0.2-μm filter before use.

### 2.2.6 Fixation of Feeder Layer

3 fixation protocols were used in the fixation of the feeder layer: 4% paraformaldehyde (PFA), 95% ethanol (EtOH) and 100% methanol (MeOH). PFA fixation, cells were fixed for 15mins at room temperature. For EtOH and MeOH fixations, cells were fixed at -20°C for 10 mins. After each fixation procedures, cells samples were washed 3X with PBS at room temperature.

## 2.3 Immunocytochemistry

Medium was removed and the cells fixed for 15 mins in cold 4% PFA / PBS followed by two PBS washes. Cells were permeabilised with 0.15% Triton X100 in PBS for 15 minutes and non- specific binding was blocked with 10% normal goat serum (NGS, Vector labs) in PBS for 1 hour at room temperature. Primary antibodies were incubated overnight at 4°C. After 3 washes at 5 mins each, secondary antibodies were incubated at room temperature for 2 hrs.

Samples were then washed 3 times at 5 min intervals and the nucleus were stained for DAPI (60sec) (Table 1). After the appropriate washes, cells in chamber slides had the chambers dislodged, and mounted with Hydromount (National Diagnostics) and a #1 24x60mm coverslip (Menzel-Glaser). Cells cultured in cell-culture plates were washed and kept in PBS at 4°C.

## 2.4 Imaging

Mounted slides were analyzed using a Zeiss AxioImager Z1 (Carl Zeiss Inc, USA) up-right fluorescent microscope. Cells in cell culture plates were analyzed using a Zeiss AxioImager Z1 (Carl Zeiss Inc, USA) inverted fluorescent microscope.

Calcium imaging experiments were carried out with a Nikon TI-E Inverted Microscope and images were captured with a CoolSNAP HQ2 camera (Photometrics, USA). Images were acquired and analyzed using the Nikon NIS-Elements software. The fluorescent $Ca^{2+}$ indicator used was rhod-2, AM (Invitrogen) at 60μM. Cells were incubated at 37˚C with the dye for 30 mins. After 3 washes, cells were incubatored for 1 hr at 37˚C before the experiment. 100 μM of KCl was utilized to stimulate cell. KCl was directly put into the culture dish.

## Primary Antibodies

| **Antigen (species directed against)** | **Isotype Host** | **Manufacturer** | **Working concentration (dilution factor)** |
|---|---|---|---|
| Anti-Nestin, clone 10C2 | IgG1 Mouse | Chemicon, MAB5326 (monoclonal) | (1mg/ml) 1:200 |
| Anti-β-III-Tubulin | IgG1 Mouse | Chemicon, MAB1637 (monoclonal) | 1 mg/ml (1:100 to 1:500) |
| Anti-β-III-Tubulin – Tuj 1 Clone | IgG1 Mouse | Covance (monoclonal) | 1:300 |
| Anti-Sox2 | Rabbit | Chemicon, AB5603 (polyclonal) | 1 mg/ml (1: 300) |
| Anti-PSA-NCAM | IgM Mouse | Chemicon, MAB5324 | 1:200-1:400 |
| Anti-TH | Rabbit | Pal-Freeze | 1:500 |
| Anti-Ki 67 | Rabbit | Chemicon, MAB4190 (polyclonal) | 1 mg/mL (1:1000) |
| Anti-MAP2 | IgG1 Mouse | Chemicon MAB 3418 | 1:500 |
| Anti-GFP | Rabbit with A488 Conjugated | Invitrogen, A21311 | 1:1000 |
| Anti-GFAP | Rabbit | Chemicon, AB5804 (polyclonal) | 1:1000 |
| Anti-DCX | Rabbit | Cell Signalling, 4604 (polyclonal) | 1:200 |
| DAPI | - | Sigma, D417 | 1X |

## Secondary Antibodies

| Antigen (species directed against) | IsotypeHost | Manufacturer | Working concentration (dilution factor) |
|---|---|---|---|
| Alexa488 (ms) | IgG (H+L) goat | Invitrogen, A11001 | 1:800 |
| Alexa488 (rbt) | IgG (H+L) goat | Invitrogen, A11008 | 1:800 |
| Alexa488 (ms) | IgG (H+L) donkey | Invitrogen, A-21202 | 1:800 |
| Alexa555 (rbt) | IgG (H+L) goat | Invitrogen, A21429 | 1:800 |
| Alexa594 (rbt) | IgG (H+L) goat | Invitrogen, A11037 | 1:800 |

**Table 1: Antibody List.** Primary and secondary antibodies used and their respective dilutions.

## 2.5 Cell Sorting

GFP tagged NSCs were sorted using FACS (BD, USA). Wild type (wt) NSCs were used as control to set the threshold and cells with intensity above the control were sorted as positive.

## 2.6 Electrophysiology

Electrophysiological experiments were done in collaboration with A/P Soon Tuck Wah at the department of Physiology, NUS. In brief, action potentials were recorded with the current-clamp whole-cell configuration. The pipette solution for current-clamp experiments contained (in mM): 123 K-gluconate, 10 KCl, 1 MgCl2, 10 HEPES, 1 EGTA, 0.1 CaCl2, 1 K2ATP, 0.2 Na4GTP, and 4 glucose, pH adjusted to 7.2 with KOH. Membrane potentials were kept around -70mV, and step currents were injected to elicit action potential. Whole-cell (inward and outward) currents were recorded at a holding potential of -70mV; voltage steps ranging from -80mV to +90mV were delivered at 10-mV increments.

## 2.7 Blocking Experiments

Gap junctions were blocked by the combination of 2 gap junction blockers: Carbenoxolone 60uM (Sigma) and 18 β-Glycyrrhetinic Acid 75uM (Sigma)(de Rivero Vaccari, et al. 2007). N-Cadherins were blocked with an antibody for N-Cadherin, monoclonal mouse anti human N-Cadherin Clone 6G11, kappa.

## 2.8 Molecular Biology Techniques

### 2.8.1 RNA Isolation

The QIAGEN-RNeasy Mini Kit was used to extract RNA. Media was removed and cells were washed with HBSS. Cells were then incubated with RLT lysis buffer for 2 minutes, at which time cells appear to form a dense viscous liquid. Using a cell scraper, the lysed cells were transferred into the QIA shredder filter column. The rest of the steps were as instructed in the manufacturer's instructions for the extraction of RNA from animal cells.

### 2.8.2 Genomic DNA Isolation

The QIAGEN-DNeasy Mini Kit was used to extract RNA. Media was removed and cells were washed with HBSS. Cells were then trypsinized and spin down to a pallet. The rest of the steps were as instructed in the manufacturer's instructions for the extraction of genomic DNA from animal cells.

### 2.8.4 PCR

PCR reactions were carried out in 25µl volumes comprising of 1x PCR buffer with $MgCL_2$, 0.2mM dNTPs, 0.5µM of each forward and reverse primers, 1 U Tag Polymerase in PCR buffer, and 5ul $H_2O$ containing template DNA. PCR conditions varied with the primer and the template used according to the Tag Polymerase product guide. Generally, the PCR products were resolved on a 1% agarose gels for 40 mins at 100V to check for the correct amplicon size.

| Candidate Name | Primer Sequence | Amplicon Size |
|---|---|---|
| Nestin Fd NM_006617 | TTGAGACACCTGTGCCAGCCTTTC | 414 |
| Nestin Rv NM_006617 | AGATTTGCCCTTCACCTTCCC | 414 |
| Musashi Fd NM_002442 | CAGCCAAAGGAGGTGATGTC | 451 |
| Musashi Rv NM_002442 | CGCTGATGTAACTGCTGACC | 451 |
| GFAP-1 Fd NM_002055 | AATGCTGGCTTCAAGGAGAC | 406 |
| GFAP-1 Rv NM_002055 | CCAGCGACTCAATCTTCCTC | 406 |
| GAD-65 Fd NM_000818.1 | CGCCCGCAGCTCGCACTC | 380 |
| GAD-65 Rv NM_000818.1 | ACACGCCGGCAGCAGGTCT | 380 |
| TH-7 Fd NM_000360 | TCATCACCTGGTCACCAAGTT | 125 |
| TH-7 Rv NM_000360 | GGTCGCCGTGCCTGTACT | 125 |
| MAP2 Fd NM_001039538 | AGCAGCAGGTGGGGAATCA | 490 |
| MAP2 Rv NM_001039538 | CAGGTCTGGCAGTGGTTGGTT | 490 |
| GAPDH Fd NM_002055 | AGGGGTCTACATGGCAACTG | 228 |
| GAPDH Rv NM_002055 | CGACCACTTTGTCAAGCTCA | 228 |
| V-SNARE Fd NM_006555.3 | ACAGGCAGGCCCCCACTTCAG | 200 |
| V-SNARE Rv NM_006555.3 | CACACTCCCCGCCCACAATG | 200 |
| T-SNARE Fd NM_145003.1 | CGCCACCTCTGTCCGAAAGT | 222 |
| T-SNARE Rv NM_145003.1 | ACAGGGGCGAGTGGGGTCAG | 222 |
| Complexin 1 Fd NM_006651.3 | AGGCACCGGACGCTGGAGACC | 330 |
| Complexin 1 Rv NM_006651.3 | CACGCCCTGGCCCGAAACA | 330 |

**Table 2: Primer list.** Reverse Transcriptase PCR primers used for the characterization of NSC and differentiated cell cultures on the mRNA expression level

## 2.9 Making of transgenic promoter reporter cell lines: DDC / eGFP

### 2.9.1 Gateway® Cloning Technology

The Gateway® Cloning Technology platform is based on recombineering; recombination sites are used to insert sequences of interest into respective vectors that become the Entry Clone and the Expression Clone. Using PCR, respective individual sequences of interest were flanked with the respective recombination sites (attB1, attB5r, attB5 and attB2). Via the BP reaction, the sequence of interest was recombined into the pDONR vector to form the Entry Clones (pENTR). The individual pENTRs are combined via the LR reaction at site specific recombination sites into the Destination Vector, to form the Expression Clone. This Expression Clone is cloned into live cells to create the Promoter / Reporter cell line.

### 2.9.2 Creation of pENTR

In the creation of pENTR, primers for the following sequences of interest were made:

i. The promoter region for Human DDC (Dopa Decarboxylase)
ii. Fluorescent reporter constructs: eGFP

i. The human DDC promoter construct was taken from Chatelin S, et al(Glaser, et al. 2007) and extracted, via PCR amplification, from the genomic DNA of undifferentiated ReNVM cells as template. According to the Chatelin study, a 3.6kb segment of neuronal promoter region that includes neuronal exon 1 and exon 2 is necessary to represent DDC expression in neuronal tissue. The non-neuronal exon 1 is necessary for the DDC expression in non-neuronal tissues such as liver and kidney, but not necessary for neuronal DDC expression. Thus,

our construct had 3 components which contained the neuronal first alternative exon (61 bp), 3.5 kb of 59 flanking sequence (nts 157083–153528 in Genbank file AC018705), and the non-coding part of exon 2 (31bp, nts 136628–136598 in AC018705). Figure 2 shows the schematic of the DDC construct with its eGFP reporter. As the 3.5kb region and the 61bp neuronal fist exon followed in order, the attB1 primers, which were the forward primers, were used to construct the sequence. The 31bp from the non-coding exon was incorporated into the reverse primers, attB5r (See Box 1; Appendix). The PCR products were resolved by size by gel electrophorosis on a 1% agarose gel. The band at the appropriate size of 3.6kb was cut, purified and recombined (BP reaction) with the Donor vectors (pDONR) that contain the corresponding attP recombination site, thus forming the DDC promoter pENTR.

ii. The eGFP reporter construct

The eGFP fragment was extracted, via PCR, out of a commercially available eGFP plasmid, pEGFP-NI from ClonTECH (GenBank Accession # U55762, Catalog # 6085-1). The forward PCR primer contained the attB5 recombination site together with the Kozak sequence and the initial 22 nucleotides inclusive of atg (start codon). The reverse primers contained the final 20 nucleotides ending with taa (stop codon) followed by the aatB2 recombination site (See Box 2; Appendix). The PCR products were resolved by size by Gel Electrophorosis on a 1% agarose gel. The respective bands at the appropriate size of ~800bps were cut, purified and recombined (BP reaction) with pDONR that contain the

corresponding attP recombination site, thus forming the respective reporter pENTR.

### 2.9.3 Transformation of plasmid into chemically competent bacterial cells

The respective BP reaction products were transformed into One Shot® March1™ T1 competent E.coli cells. One vial (~50ul) of bacterial cells is incubated on ice with 2ul of BP recombination reaction product for 30 mins. Transformation was induced by heat shock treatment at 42° C for 30 sec. The cells were then cooled on ice for 2 mins and 250ul of pre-warmed nutrient rich LB S.O.C was added into each cells containing vial. The vial was incubated at 37°C while shaking horizontally for 1hr at 225rpm.

Following incubation, 20ul to 100ul of culture was plated onto Transformed LB-agar plates containing the respective antibiotic, in this case being Kanamycin (50ug/ml). Due to the Kanamycin resistance sequence in the pENTR vector, cells that were not transformed with the vector were killed by the drug. As a negative selection, the untransformed cells contain the CCDB gene which is toxic and will thus kill the cells. Plates were incubated overnight at 37° C to allow the growth of bacterial colonies.

### 2.9.4 Isolation and analysis of Colonies

After incubation, individual colonies were picked with a 200ul pipette tip and inoculated overnight at 37°C in 5ml of LB media with the respective antibiotic, shaking at 225rpm. The cells in suspension were then lysed and plasmid DNA purified using the Qiagen miniprep kit according to the manufacturer's instructions. The DNA was quantified with Nanodrop (ND-1000) and analyzed by doing the appropriate restriction enzyme (RE) digest. The DNA from colonies that gave the correct bands after RE digest was sent for

sequencing and the sequencing results were validated with the *in-silico* derived plasmid DNA sequence.

### 2.9.5 Creation of the Expression Clone

The positive pENTR clones of the promoter and the reporter were then recombined on the backbone of the Destination Clone via the LR reaction, to form the Expression Clone. The LR reaction was done as per the manufacturer's instructions and separate reactions were done to create the DDC-eGFP Expression Clone.

## 3.0 Statistical Analysis

All statistical analysis was performed using GraphPad Prism 5.03. When comparing two groups of data, the paired t-test was used. When comparing groups of data, one-way ANOVA was used to check if the within a population was significant. Then, the Dunnett Post Hoc test was used to compare individual samples within the population. The Dunnett's Post Hoc test compares the means against a single control, after ANOVA shows non-equality or differences between groups.

(* represents $p<0.05$) (** represents $p<0.01$) (*** represents $p<0.0001$)

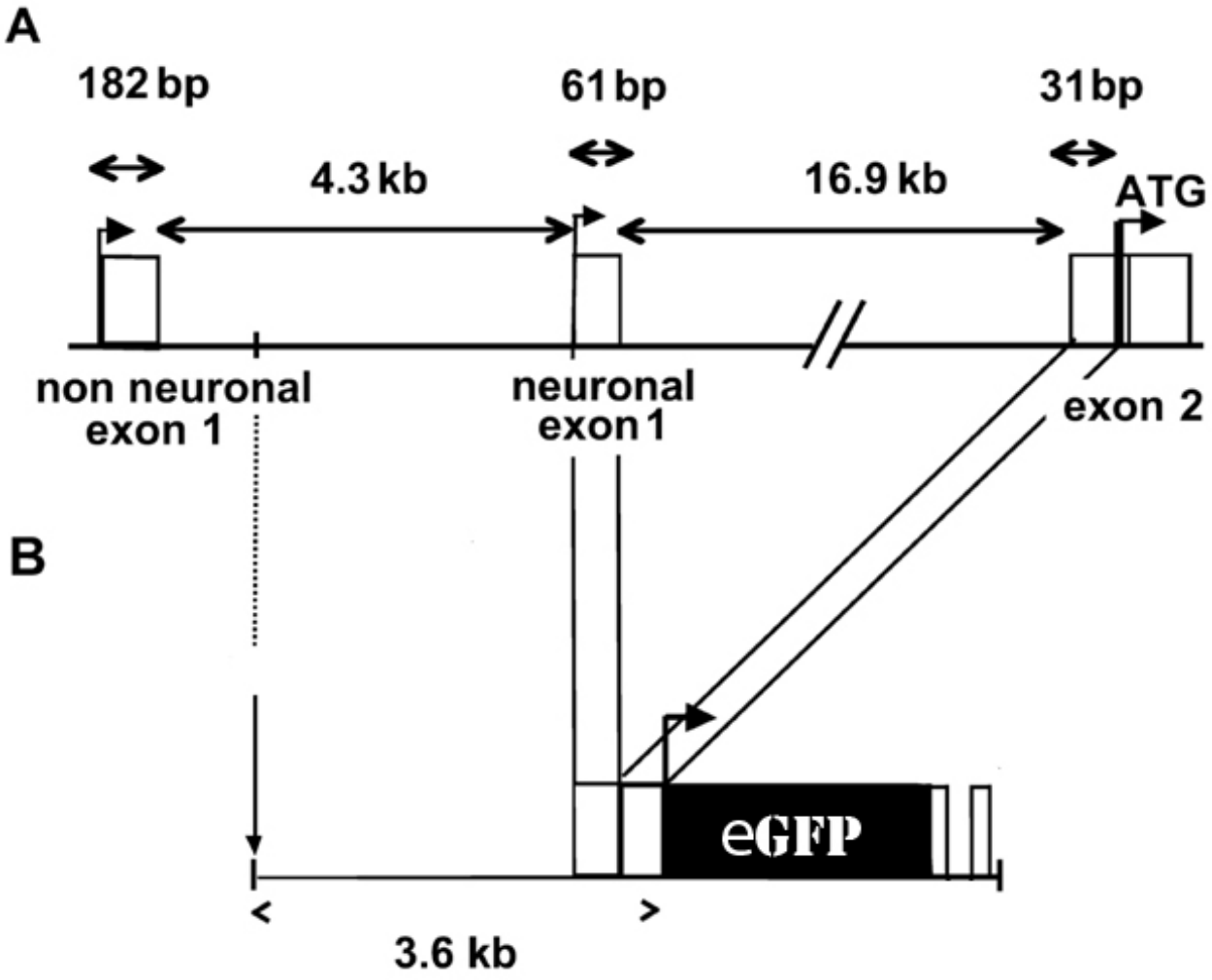


**Figure 2: Schematic diagram showing the optimized DDC-GFP vector.** (A) Represents the promoter region and the coding region of the DDC gene. (B) The optimized promoter region joins only part of the non-neuronal exon 1, neuronal exon 1 and neuronal exon 2. This sequence is flanked by the eGFP sequence. (Modified from **(Chatelin 2001)**

# Chapter 3:

# The differentiation of neural stem cells (NSCs) using an adherent monolayer cell culture system

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### 3.1 Introduction

Though human Neural Stem Cells (hNSCs) can be isolated or generated from different cellular sources (Fig. 1), they are generally cultured in a standard fashion. NSCs are generally propagated in the presence of mitogenic factors such as epidermal growth factor (EGF) and/or fibroblast growth factor 2 (FGF2) either as neurospheres (clusters of cells in suspension attached to each other) or as an adherent mono-layer of cells seeded on a protein coated surface (Glaser, et al. 2007; Gritti Angela, et al. 2001). The mitogenic growth factors keep cells in the cell cycle, and thus supports proliferation and self-renewal of NSCs. NSCs grown as neurospheres have shown to be disadvantageous because they contain a mixture of neural stem cells, more differentiated progenitor cells as well as differentiated neurons and glia in a common ECM (Reynolds and Rietze 2005; Campos, et al. 2004; Glaser, et al. 2007). Clonal analysis of cells from dissociated neurospheres revealed that only a small percentage (3–4%) of the cells within the sphere were truly multipotent neural stem cells (Gritti Angela, et al. 2001; Glaser, et al. 2007). As adherent cells have a more uniform distribution of NSCs, it is our choice culture method.

The human NSC line used in this study (ReNVM) is from a commercial source, derived from a 10 week old fetus (Donato, et al. 2007). After propagation and upon the removal of the mitogenic growth factors, cells spontaneously but gradually exit the cell-cycle and differentiated to give rise to 3 different neural lineages such as astrocytes, oligodendrocytes and neurons (Reynolds and Weiss 1992; Reynolds and Weiss 1996;

Glaser, et al. 2007; Gritti Angela, et al. 2001). In this chapter I show that the NSC line maintained a tripotent differentiation capability characteristic of NSCs. I furthermore characterized the differentiation profile of NSCs in a time frame of up to 7 days by using a specific stem cell marker (nestin), a cell cycle marker (Ki 67), an intermediate neuronal differentiation marker (β-III-Tubulin) as well as a mature phenotypic marker for dopaminergic neurons (TH - tyrosine hydroxylase). I also investigated whether neurons were indeed functional by the imaging of calcium signals upon depolarization. 100mM of KCl was used to depolarize cells. The addition of $K^+$ ions into the extracellular space creates a concentration difference between the intracellular and extracellular $K^+$ concentration and thus cause the diffusion of $K^+$ ions into the cell. Due to the minuscule volume of the cell, a very small concentration of $K^+$ ions is sufficient to dramatically increase its membrane potential causing a membrane depolarization (Bear, et al. 2007). Increase in Calcium signal upon depolarization is used as an indicator of neuronal differentiation (Zhang, et al. 2010; Piacentini, et al. 2008; Goldman 1998) and our cells were thus characterized based on their $Ca^{2+}$ signal.

A hallmark of a neuron in the CNS is its ability to communicate with other neurons via synaptic connections (Kandel, et al. 2000; Waites, et al. 2005). In brief, neurotransmitters trigger the openings of channels which allow the flux of either positive or negative ions (Bear, et al. 2007). The influx of positive ions rises the membrane voltage at the axon hillock above the threshold for firing and this triggers an action potential down the axon, which leads to the opening of voltage gated calcium channels (VGCC) (Kandel, et al. 2000). The influx of $Ca^{2+}$ ions triggers the release of neurotransmitters which in turn would trigger downstream neurons. To understand if our

cells were electrophysiologically mature enough to generate action potentials, Patch Clamp analysis was performed.

Thus in this chapter, I characterized the cultured hNSCs and their differentiation using the adherent 2-D NSC culture system on the level of marker gene expression and functionality. I show that while the system is conducive in maintaining stem cells, it is sub-optimal in yielding differentiated neurons. The time-points used were day 0 (control or undifferentiated NSCs) to day 7 of differentiation. In the $Ca^{2+}$ imaging experiments cells were assayed at day 0, day 7 and day 14. In the electrophysiological analysis, cells were analyzed at day 0 and day 14 as no significant difference from day 0 was noticed at day 7.

## 3.2 Results

### 3.2.1 Characterization of NSC: NSCs exhibit a globular morphology and express NSC markers

In the undifferentiated state NSCs have a globular morphology with a relatively large cell body and with short neurites or neurite-like extensions (Fig. 3A). These cells express NSC markers (assayed via immunocytochemistry) such as Nestin, GFAP, (Fig. 3B), SOX 2 (Fig. 3C), and Musashi 1 (Fig 3E).

Nestin is a member of the intermediate filaments protein family, which is thus far found only in vertebrates. Nestin is important for the proper survival and self-renewal of NSCs and is accepted as a distinctive NSC marker (Gilyarov 2008; Lendahl, et al. 1990; Park, et al. 2010; Okuno, et al. 2010) (Fig. 3A).

Co-labeled with Nestin in Figure 3B is GFAP. GFAP (glial fibrillary acidic protein) is generally an astrocytic marker (Grupp, et al. 2010; Lam, et al. 2009).

However, as NSCs are closely related to the radial glial lineage (Götz and Steindler 2003) GFAP is also expressed in NSCs. But its expression is lost upon neuronal differentiation, but retained upon astrocytic differentiation.

SOX 2 is a transcription factor and a member of the SOX [SRY (sex determining region Y)-box] family of genes. It has been shown to be expressed in functionally defined NSCs and it supports cells in maintaining NSC properties (Pevny and Nicolis 2010; Brazel, et al. 2005) (Fig. 3B). As such, it is also used as a NSC marker.

As NSCs are active in the cell-cycle, they also express Ki67 (Fig. 3C) a cell cycle marker, present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from (G0) resting cells (Scholzen and Gerdes 2000). At different phases of the cell-cycle, Ki67 is expressed at different regions within the nucleus (Scholzen and Gerdes 2000).

Musashi 1 is a neural RNA-binding protein that is strongly expressed in fetal and adult NSCs (Fig. 3D). However, they also have a role in epithelial stem cells, including intestinal and mammary gland stem cells and thus need to be used in combination with other NSC markers (Okuno, et al. 2010). In this study, Musashi expression is shown on the mRNA expression level.

NSCs exhibited a globular morphology and most cells did not posses detectable neurites or neurite- like protrusions (Fig. 3A). Such NSCs expressed stem cells markers such as Nestin (Fig. 3B), SOX 2 (Fig. 3C), GFAP (Fig. 3 B & C), Musashi (Fig. 3E) and as they were active within the cell-cycle, they expressed Ki67 (Fig. 3D). Thus, based on this evaluation cells were regarded as bona-fide NSCs.

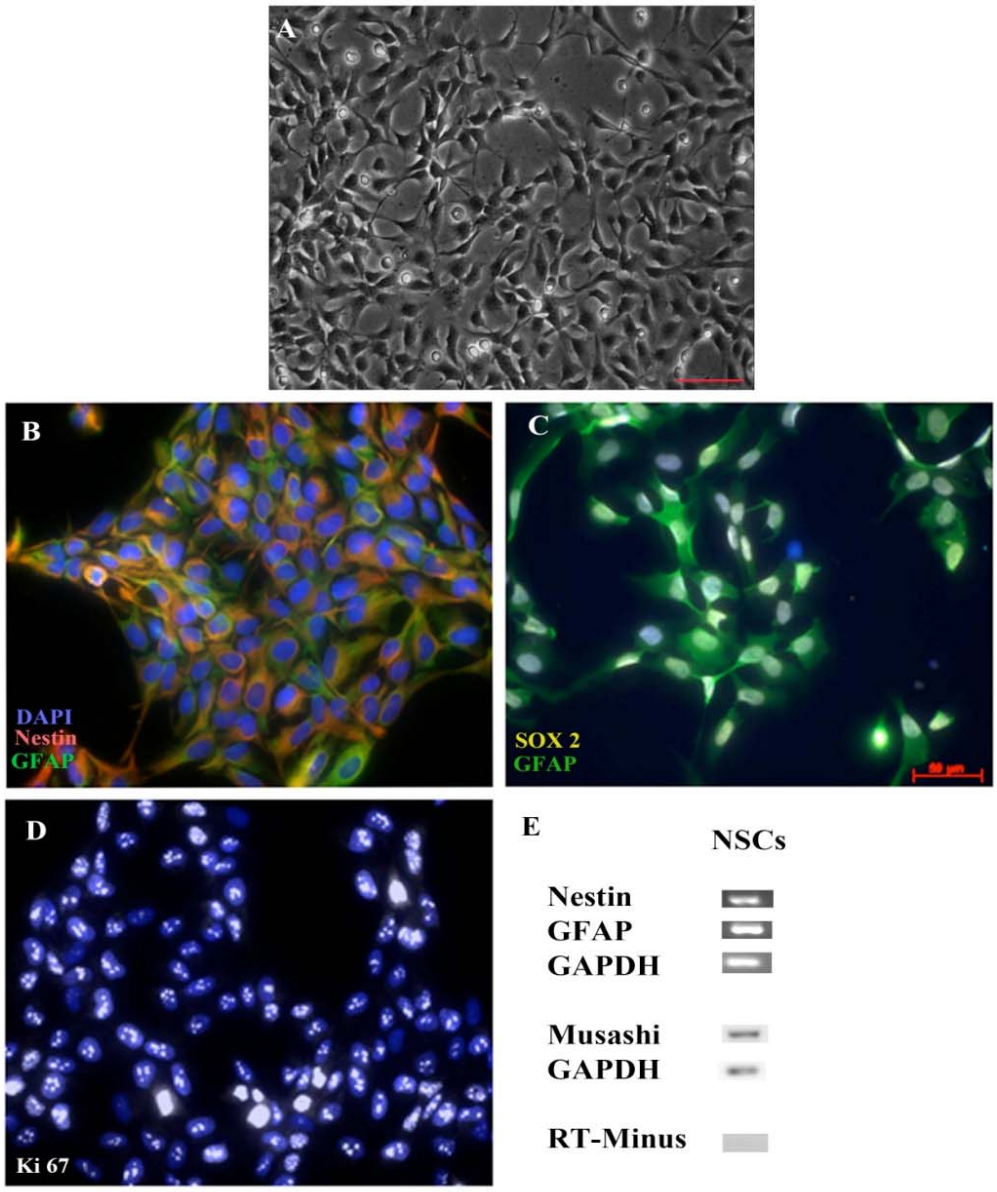


**Figure 3: Characterization of NSCs.** (**A**) Bright-field image of proliferating NSCs. When grown in media containing growth factors (EGF and bFGF) cells posses a globular morphology with a relatively large cell body. Scale bar = 100µm. (**B-D**) show immunocytochemical analysis of stem cell related markers. Scale bar = 50µm **(B)** Cells express NSC markers Nestin (red) and GFAP (green). (**C**) NSCs also express SOX 2 (yellow), a nuclear localized marker. Note that all NSCs co-labeled with SOX2 and GFAP. (**D**) Ki 67 (white), a marker of cells in the cell-cycle is expressed in all NSCs, indicating their proliferative nature. (**E**) The expression of NSC markers (Nestin, GFAP and Musashi) was also tested at the mRNA expression level. The expression of nestin, GFAP and Musashi was detected in NSCs. GAPDH, a house keeping gene was used as the loading control.

### 3.2.2 NSCs spontaneously differentiated, exhibited a differentiated morphology and expressed mature neural lineages markers

When mitogenic growth factors were removed, NSCs over time exit the cell cycle and spontaneously exhibited a more mature differentiated morphology with extended neurite-like protrusions (Fig. 4A). The red arrows point toward some of the elongated protrusions.

Within 7 days of removing mitogenic factors (bFGF & EGF), cells spontaneously differentiated into the different neural lineages: neurons (Fig. 4A), astrocytes (Fig. 4B), and oligodendrocytes (Fig. 4C)

Cells expressed markers for immature neurons, doublecortin (DCX) and β-III-Tubulin (Fig. 4B). Neuronal precursors begin to express DCX shortly after exiting the cell cycle, and continue to express DCX till the cells mature into neurons before being down regulated (Bernal and Peterson 2011). β-III-Tubulin is a neuron specific isotype of β-Tubulin, that is phosphorylated when NSCs are induced to differentiate (Peters 1960). Cells also expressed the mature neuronal marker MAP2 (Fig. 4E), which is a neuron-specific cytoskeleton protein enriched in dendrites (Sims, et al. 1988). Furthermore, tyrosine hydroxylase (TH) (Fig. 4F), a critical enzyme involved in the synthesis of the neurotransmitter dopamine (Cho, et al. 2008; Vitalis, et al. 2005; Friling, et al. 2009; Siegel and Agranoff 1999), was expressed in ~0.5% of spontaneously differentiating cells indicating the potential of these cells to differentiate into dopaminergic neurons. TH as well as dopamine decarboxylase (DDC), another marker for DA neurons, was also expressed on the mRNA level (Fig. 4G). In addition, cells up-regulated the expression of GAD65 which is indicative for GABAergic neurons (Erlander, et al. 1991). However, as I did not observe any regulation of other neurotransmitter markers such as tryptophane

hydroxylase (TPH; seretonergic marker), choline acetyl transferase ChAT (cholinergic marker) and dopamine-β-hydoxylase (DBH; norepinepharinergic and epinephrinergic markers; (McClellan, et al. 1994))(data not shown), it suggests that the NSCs predominantly differentiated into GABAergic and dopaminergic neurons.

NSCs also differentiated into astrocytes (Fig 4D) & oligodendrocytes (Fig 4E), which comprise 2 major subpopulation of glial cells (Demir, et al. 2009; Adelman and Smith 1999; Kriegstein and Alvarez-Buylla 2009; Götz, et al. 2002). As mentioned above, astrocytes like NSCs, express GFAP and have a star like morphology (Fig. 4D). These cells act as supporting cells in the CNS. Oligodendrocytes, which express O1, form myelin sheets that ensheathe axons, thus enabling saltatory conduction of action potential (Demir, et al. 2009).

Apart from general marker gene expression, neuronal cells should possess the necessary 'machinery' to communicate with other neural cells, by forming active synapses. The presence of some synaptic markers such as T-SNARE, V-SNARE and complexin (Fig. 4H), seen on the RNA expression level, may hint that these cells have the ability to form active synapses (Carr and Munson 2007). On the expression level, it should also be noted that some markers, such as TH, V-SNARE and complexin are expressed even in the undifferentiated NSC. Though this was not further investigated, it could be speculated that the message was present even in the undifferentiated state but needed developmental cues before becoming functionally active proteins.

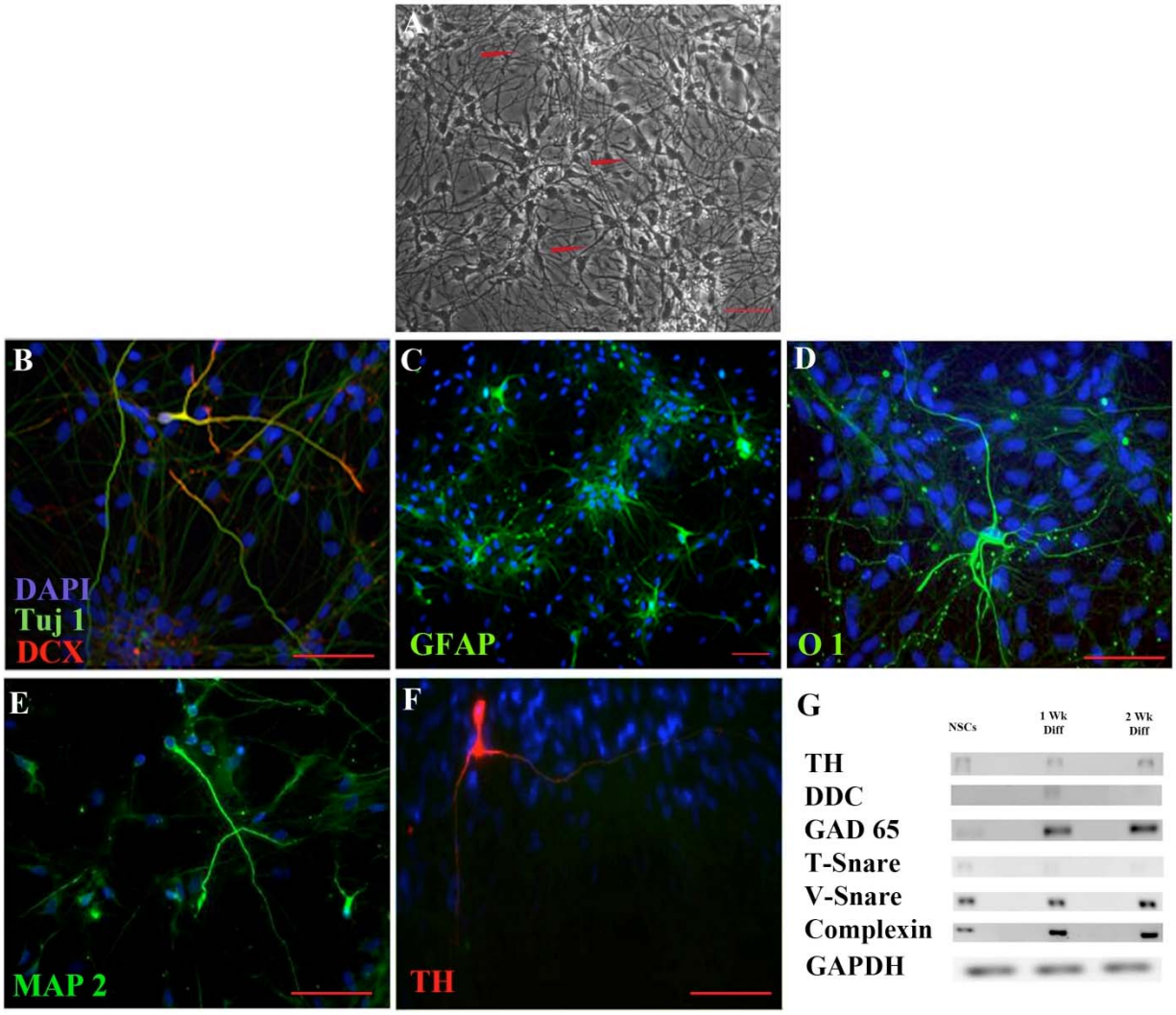


**Figure 4: Characterization of differentiating NSCs at day 7 after the removal of mitotic factors (FGF2 & EGF).** (**A**) Cells show an elongated morphology with relatively smaller cell bodies and extended neurite-like extensions (red arrow). Each extension possesses multiple sub-branches. (**B-F**) shows the immunocytochemical analysis of differentiation markers. (**B**) Cells express immature neuronal markers such as doublecortin (DCX) and β-III-Tubulin, (**C**) the astrocytic marker GFAP and (**D**) the oligodendrocyte marker O1. Some cells express more mature markers such as (**E**) MAP2, a mature neuronal marker and (**F**) TH, a phenotypic marker for dopaminergic neurons. (**G**) From the RT-PCR analysis, phenotypic markers such TH and DDC are expressed as well as GAD65, possibly indicating the presence of dopaminergic neurons and GABAergic neurons respectively. Synaptic markers such as T-SNARE, V-SNARE and Complexin are also seen on the expression level although these messages are already expressed in the undifferentiated stage. GAPDH was used as a loading control. Scale bar = 50μm.

### 3.2.3 Temporal characterization of differentiation

In order to characterize the temporal profile of NSC differentiation, cultures were assayed for the percentage of Nestin expression as a stem cell markers, Ki 67 expression as a proliferation marker, β-III Tubulin and TH expression as a phenotypic dopaminergic neuron differentiation marker on the level of immunocytochemistry. NSCs were propagated and upon deriving the appropriate number of cells, mitotic factors were removed and NSCs spontaneously differentiated. Cells were assayed daily for the above markers from day 0 of differentiation to day 7 of differentiation.

We observed that though a decline in the expression of Nestin was seen as NSCs progressed into differentiation, Nestin generally continued to be expressed strongly in the culture over 7 days of differentiation (Fig. 5). As Nestin is a NSC marker, it is counter intuitive that Nestin continued to be expressed after 7 days of differentiation. The expression was then quantified according to the percentage of total cells expressing Nestin (Fig. 5A). Though the Nestin expression is significantly less after 3 days of differentiation ($p < 0.001$), more than 80% of cells continue to express Nestin up to 7 days of differentiation. To validate this point (that NSC markers were expressed after days of differentiation), SOX 2 was assayed. Though data is not shown, in concurrence with Nestin expression, SOX 2 continued to be expressed even after 14 days of differentiation. Thus, within this culture system, the NSCs used continue to express NSC markers Nestin and SOX 2 even after the induction of differentiation.

However, the proliferation marker Ki 67, sharply declined between days 3 and 4 and was virtually absent from the cultures at day 7 (Fig 5 & 6B). Taken together, this set

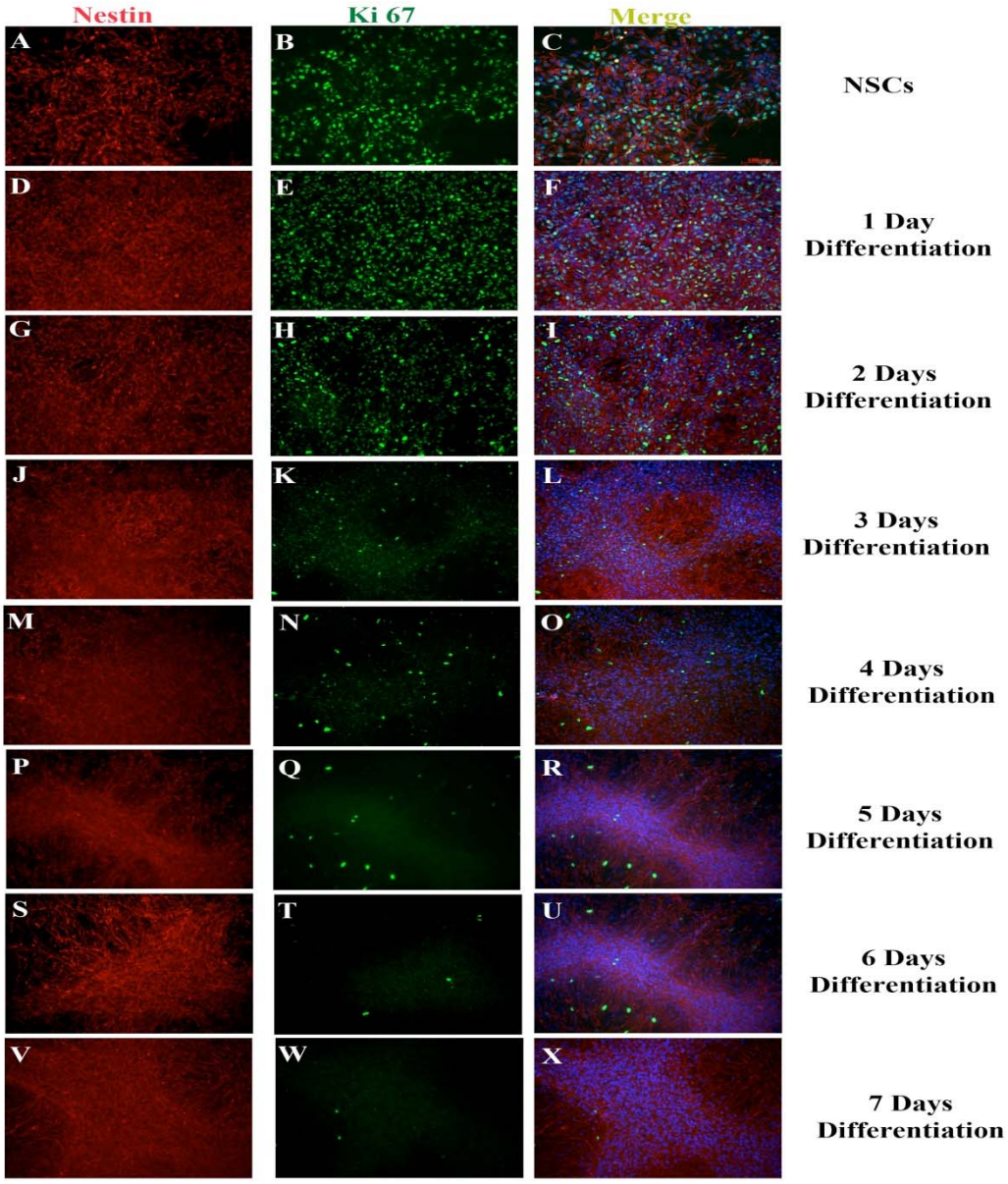


**Figure 5: Characterization of the NSC marker-Nestin and the cell-cycle marker Ki 67 via immunocytochemistry over 7 days of differentiation.** The column of images on the left represents the expression of Nestin (red). The middle column represents the expression of Ki 67 (green). The column on the right is the merged column including the nuclear marker DAPI (blue). Nestin continued to be highly expressed throughout the 7 days of differentiation with expression seen in the undifferentiated cells (**A**) and in the 7 days differentiated cells (**V**). But the general staining pattern in the frame was different in the differentiated cells as compared to the non-differentiated cells. Ki 67, seems to be down regulated over time. Its expression is high between days 0 (**B**) and day 2 (**H**) of differentiation. However, progressively lesser cells express Ki 67 between day 3 and day 7 of differentiation. Thus, some cells, although being positive for nestin are not actively cycling. Scale bars = 100um.

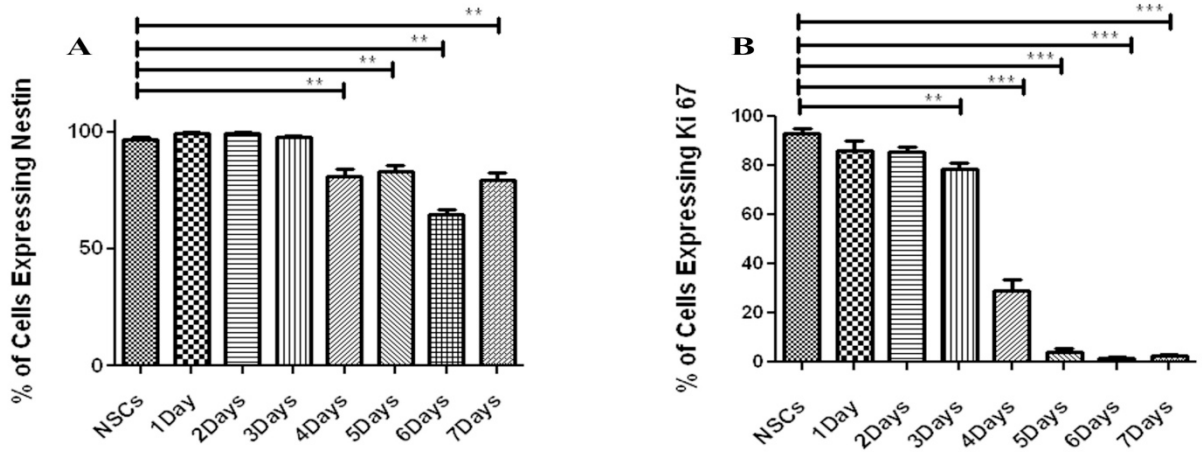


**Figure 6: Quantification of Nestin and Ki 67 expression during 7 days of differentiation. (A)** Though Nestin continues to be expressed throughout the culture duration, there is some level of downregulation from 100% to 82% after 4 days of differentiation ($p < 0.01$). (**B**) Ki 67 is progressively and significantly down regulated ($p < 0.01$) after 3 days of differentiation and is virtually absent from day 6 onwards.

of data suggested that although most of the cells were not actively proliferating by day 4 into differentiation, NSCs still largely maintained the expression of NSC markers.

The analysis of the expression of neuronal markers (Fig. 7 & Fig. 8) revealed that a small number of total cells in the control condition (undifferentiated) expressed neuronal markers. This could indicate that spontaneous differentiation is already happening even when cells are exposed to mitogenic stimuli. However, from day 3 onwards the expression of the neuronal marker Tuji1 increased until reaching a plateau at day 5 and 6 when about 12% of total cells expressed the neuronal markers. Strikingly, at day 7 a sharp decrease in neuronal cells was observed possibly as a consequence of neurons undergoing cell death. Using TH as a phenotypic marker for DA neurons, we did not observe any significant increase of DA neurons in the time course of 7 days. It was observed that the maximum percentage of TH expression is ~ 0.3% - 0.5% and no significant difference is seen between the different time points of differentiation. Though other experiments that I have conducted with this same cells indicate that TH expression increases up to ~1%, all studies show no significant differences in TH expression as the NSCs differentiate indicating that a larger sample size is necessary due to the small TH population.

Thus, taken together, the data suggests that cells although they exhibit a neuronal morphology and do not actively cycle, they do not necessarily differentiate as seen with the presence of NSC markers and the low numbers of neuronal markers.

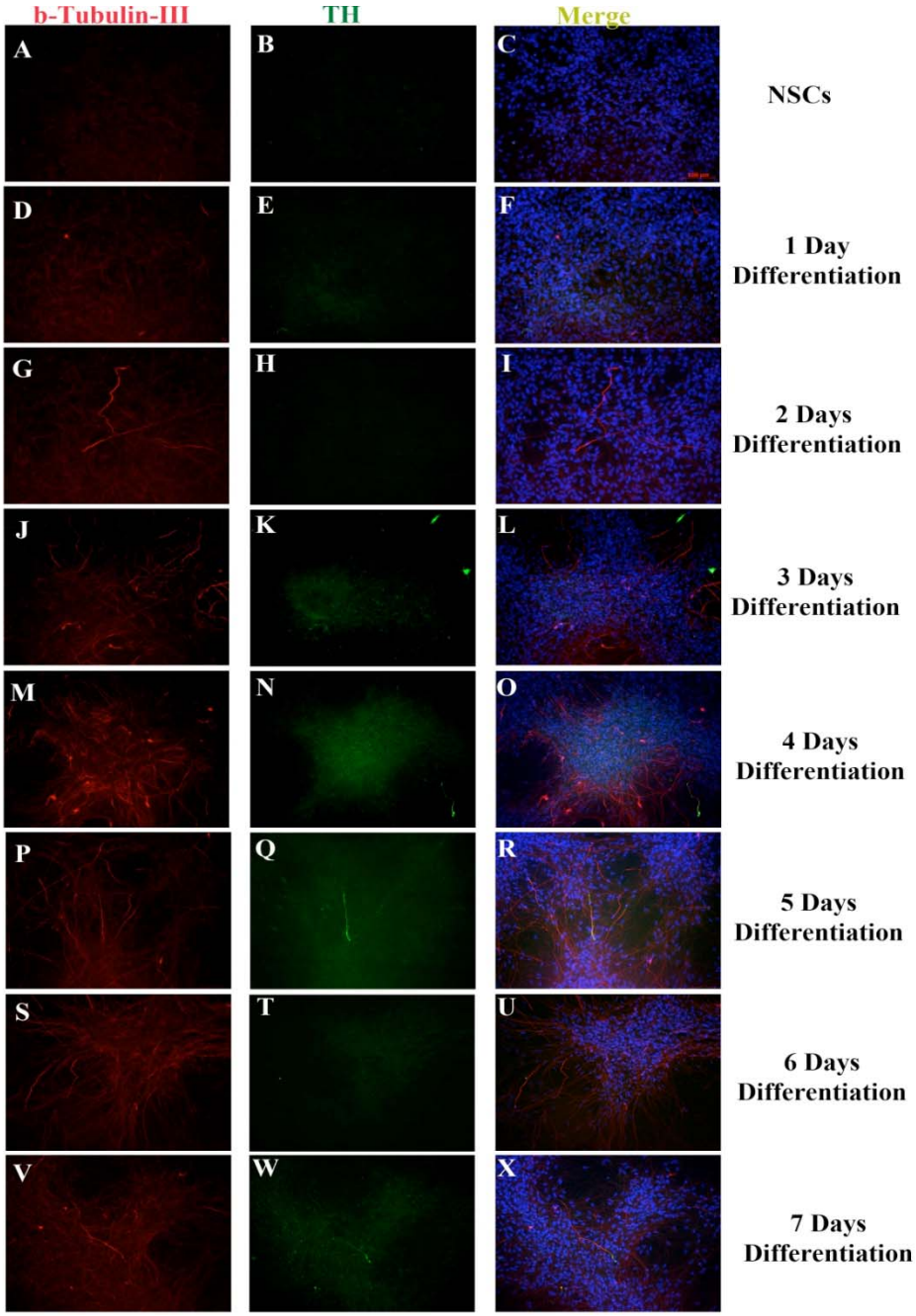


**Figure 7: Characterization of the neuronal markers - β-III-Tubulin and TH via immunocytochemistry over 7 days of differentiation.** The column of images on the left represents the expression of β-III-Tubulin (red). The middle column represents the expression of TH (green). The column on the right is the merged column with the additional of DAPI (blue) label. β-III-Tubulin begins to be expressed at ~ 3 days of differentiation (J) and the number of cells expressing β-III-Tubulin increase during further differentiation. TH expression is also observed at day 3, however lesser cells express TH as compared to β-III-Tubulin**.** TH positive cells are co-labeled with β-III-Tubulin (P-Q, V-W), indicating that some immature neurons have acquired some features of neurotransmitter maturation.

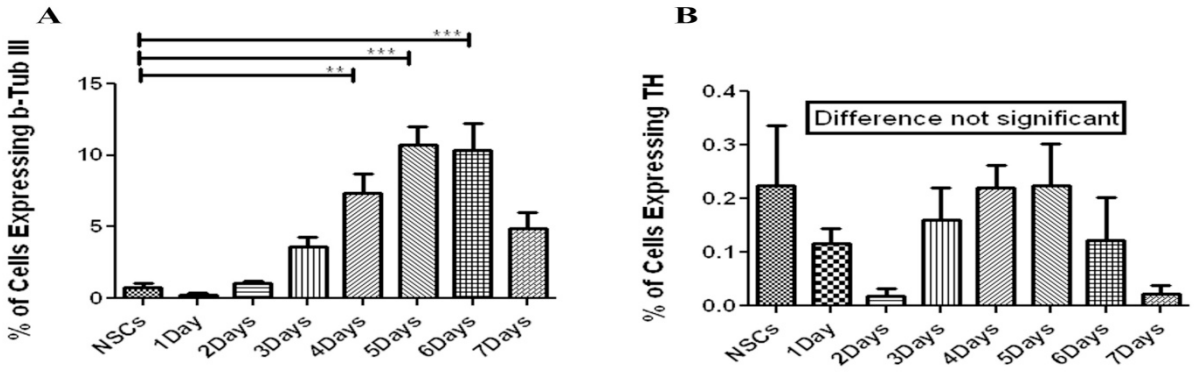


**Figure 8: Quantification of β-III-Tubulin and TH expression during 7 days of differentiation.** (A) About 0.7% of cells in the NSC population (control) express β-III-Tubulin, thus indicating spontaneous expression of a differentiation marker in NSCs. The level of β-III-Tubulin expression increases to about 11% at 5 days of differentiation. At day 7 the number of β-III-Tubulin expressing cells decrease to ~5% at 7 days of differentiation. (B) The mean of TH expression does not exceed beyond ~ 0.2% throughout differentiation. These differences are statistically insignificant.

### 3.2.4 Calcium profile of spontaneously differentiating NSCs

It has been shown that NSC differentiation correlated with the expression of voltage gated $Ca^{2+}$ channels (Bito, et al. 1997; D'Ascenzo, et al. 2006; Piacentini, et al. 2008; Fetcho and O'Malley 1997). $Ca^{2+}$ influx through these channels plays a key role in neuronal differentiation or maturation and active neurons have large increases in $Ca^{2+}$ signal upon depolarization (Goldman 1998). Thus, to characterize whether cells functionally differentiated in terms of $Ca^{2+}$ dynamics, fluorescent $Ca^{2+}$ indicator, rhod-2, AM, was used as a tool for the imaging and recording of $Ca^{2+}$ intensity in control and in differentiating cells. The control cells consisted of undifferentiated NSCs and the differentiation time-points used were 7 days and 14 days.

Figure 9A shows an example of the cells under a bright field microscope. From Figure 9B, the basal $Ca^{2+}$ intensity in the cells and the extracellular region is seen. Within cells, selected areas were chosen for intensity measurements. The $Ca^{2+}$ intensity was measured as a normalized ratio in accord to the following equation (Bito, et al. 1997; Takahashi, et al. 1999; Piacentini, et al. 2008):

$\Delta F/F$ = ($Ca^{2+}$ intensity value after stimulation – Background $Ca^{2+}$) ÷

($Ca^{2+}$ intensity value before stimulation – Background $Ca^{2+}$)

Figure 9C shows the effect of KCl stimulation on undifferentiated NSCs. Upon stimulation, a unanimous decrease in $Ca^{2+}$ intensity from ~ 1 to ~ 0.7 within all the cells is measured, thus indicating an efflux or a loss of $Ca^{2+}$ ions from the region of the cell body. At 7 days of differentiation, there is a dip (from 1 to ~0.8) after KCl stimulation (Fig. 9D).

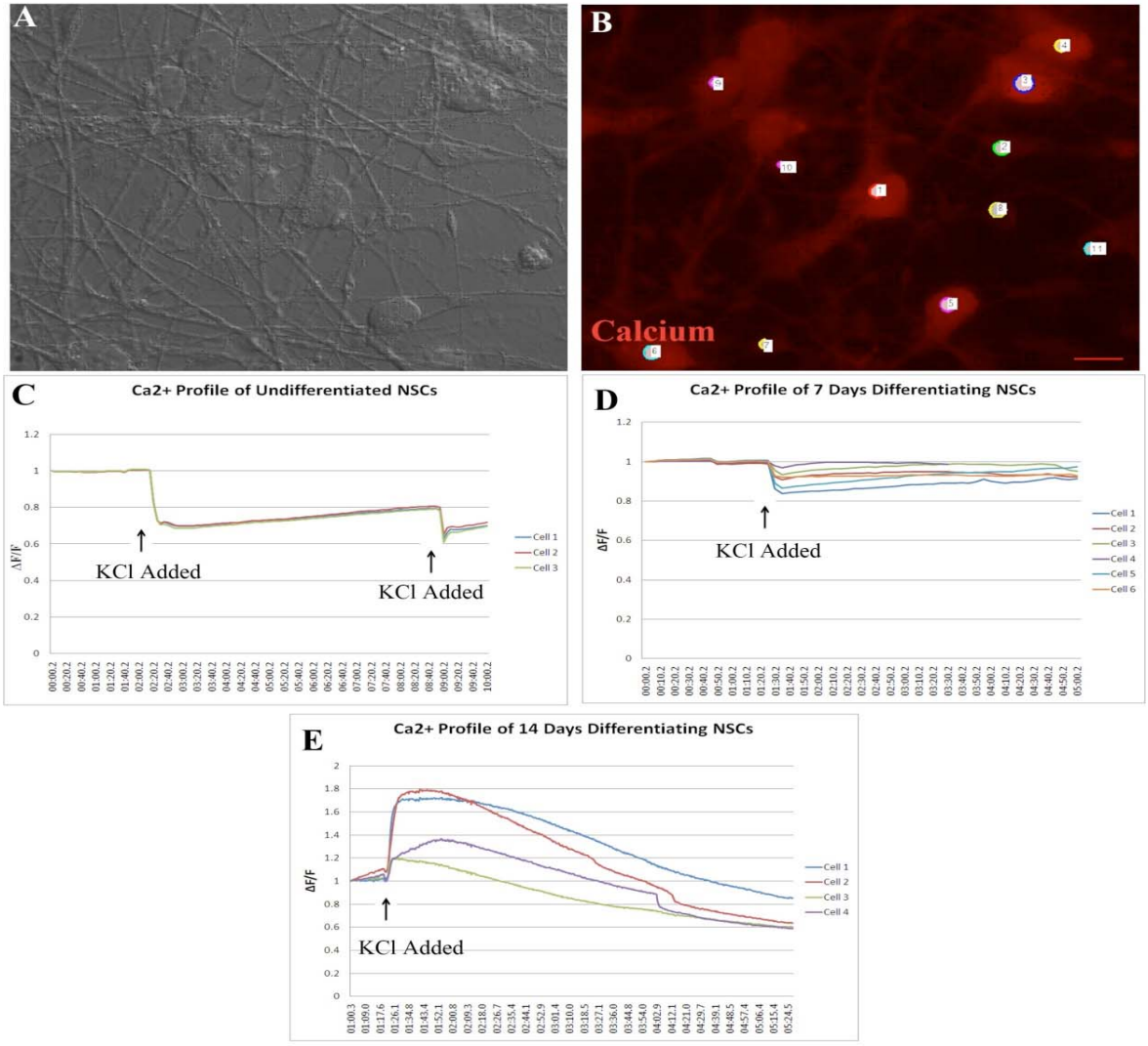


**Figure 9: Characterization of differentiation via change in $Ca^{2+}$intensity. (A)** Bright field image showing 7 days differentiated cells. The globular shapes (black arrows) represent the cell bodies and the elongated structures (white arrows) represent neurites. (**B**) Fluorescent image of the same frame seen in (**A**). The red staining represents basal level of calcium detected in cells. Cells were selected and probed at the specific regions selected on individual cells (numbered circles) for their intensity of calcium. Calcium imaging of cells was done at the different time-points (0 days, 7 days and 14 days of differentiation). (**C-E**) represent the individual time points studied: (**C**) undifferentiated cells, (**D**) 7 days differentiated and (**E**) 14 days differentiated cells. The (y) axis represents ΔF/F, which is the change in $Ca^{2+}$ intensity after stimulation. (**C**) Upon the addition of KCl, the calcium intensity within undifferentiated NSCs was reduced. (**D**) However, when KCl was added to cell after 7 days, the amplitude of the reduction in calcium intensity is lower. (**D**) After 14 days of differentiation, most cells showed an increase in calcium signal. An increase in $Ca^{2+}$ intensity has been shown to be characteristic of differentiated neurons. However, variability is seen between different cells. Scale bar = 25µm.

Furthermore, cells probed showed a difference in response, indicating a heterogeneous cell population. At 14 days of differentiation, the $Ca^{2+}$ changes with a positive slope, indicating an increase of $Ca^{2+}$ signal (Fig. 9E). All cells did not have the exact same response as the different cells had different amplitudes of signal. In some cells, the signal increases from 1to 1.8, while in other cells, it increased to 1.2 (Fig. 9E). Thus, there is either an influx of $Ca^{2+}$ ions into the cell body, or a release of $Ca^{2+}$ from the intercellular $Ca^{2+}$ stores. As in figure 9D, the different response within cells possibly indicates heterogeneity in the cells probed.

### 3.2.5 Electrical properties of spontaneously differentiating NSCs

To test if NSCs developed the maturity to trigger action potentials, in collaboration with A/Professor Soong Tuck Wah's lab at the Department of Physiology in NUS, Patch Clamping experiments were conducted. NSCs were compared with 14 days differentiating cells (Fig. 120). The whole-cell voltage was recorded by a 100-ms stimulus with a current clamp between -30 to 80 pA. Though differences were observed in the profile of the voltages generated between the NSCs and the 14 days differentiating cells, no action potentials were evoked.

The whole-cell current was recorded by a 100-ms stimulus with a voltage clamp between -30 to 90 mV in 10 mV increments, and at the holding potential of -70 mV. Again, results indicate that differences were seen between the current profile of the NSCs and the differentiating cells; the current amplitude of the differentiating cells is greater. However, in both cases, almost no inward current is observed. The inward current is usually dominated by sodium flux and it is also responsible for the rising phase of the action potential.

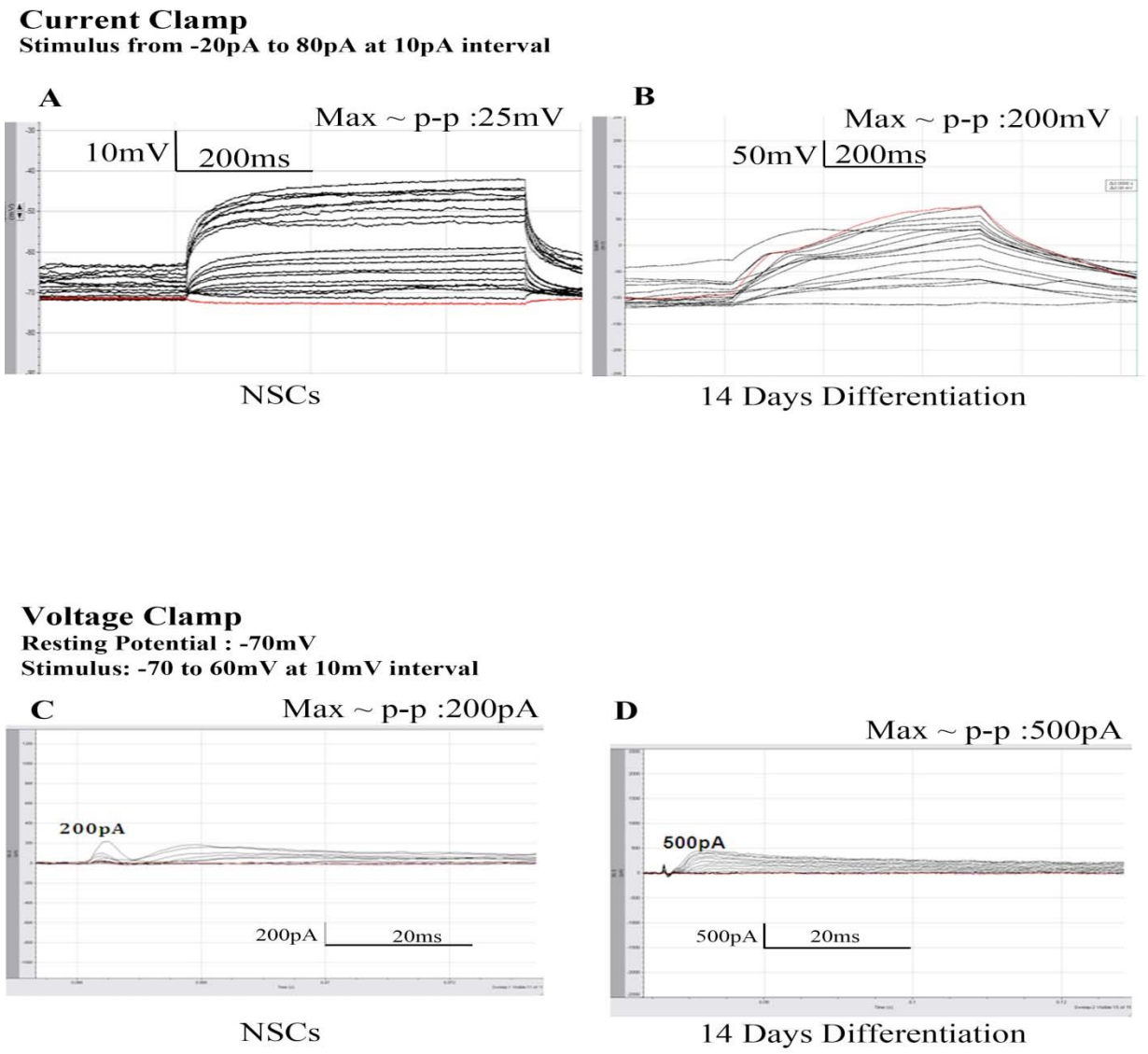


**Figure 10: Electrophysiological characterization of NSCs during differentiation.** The whole cell configuration was used to understand if cells elicit action potential. (**A, B**) show cells in the current clamp configuration. (**A**) Represents undifferentiated NSCs and (**B**) represents 14 days differentiated cells. Though the amplitude of the voltage response in the differentiating cells were approximately 8 times higher than the NSCs, an action-potential could not be observed in both conditions. (**C, D**) show cell in a voltage clamp configuration. (**C**) Represents undifferentiated NSCs and (**D**) represents 14 days differentiated cells. Similar to the current clamp configuration, differences were seen in the outward current amplitude between NSCs and 14 days differentiated cells. But as inward currents were not visible, cells do not possess the electrophysiological profile of mature neurons.

Though this was not further studied, it is possible that the lack of sodium inward current could be responsible for the lack of action potential (Bear, et al. 2007).

### 3.3 Summary

Taken together, the data shows that the standard cell culture method does not produce high yields of differentiated and functional neurons. NSC as well as a proliferation marker is maintained even under differentiation conditions. In addition, only a small percentage of cells express neuronal differentiation markers. Furthermore, though cells become electrically active as they proceed toward differentiation, they are inept in generating action-potentials and thus developing into functional neurons. This may indicate an inherent insufficiency within the culture system possibly incapable of providing the fundamental cues that are necessary to instruct all the cells to exit the stem cell mode and progress with differentiation. Thus, there is a need to engineer a system so we may get the down-regulation of stem cell markers and an increased expression of neuronal markers.

# Chapter 4:

## NSCs co-cultured with predifferentiated cells show accelerated and enhanced neuronal differentiation

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### 4.1 Introduction

During development, NSCs are generally required to make 3 fundamental decisions before they generate post-mitotic neurons (Panchision and McKay 2002; Ståhlberg, et al. 2011). Firstly, they determine their positional identity based on extrinsic signals. Secondly, based on their positional identify they need to decide whether to undergo self-renewal or mitotic arrest. Thirdly, they need to interpret mitotic arrest based on intrinsic information and extrinsic cues that directs its particular fate. During development or in adult neurogenesis, as NSCs migrate out of the stem cell niche, they are exposed to dynamic extrinsic cues which direct them to making these fundamental decisions. These extrinsic cues are dynamic and they include biochemical signals (diffusible factors), ECM interaction, cell-cell interaction with cells of different lineages and at different stages of development and physical factors. However, during *in-vitro* differentiation, the standard cell-culture system does not provide cells with these extrinsic stimuli. I hypothesized that if these conditions could be replicated in an *in-vitro* NSC culture system, it would more closely resemble the *in-vivo* environment and thus the neuronal differentiation of NSCs would be more efficient and effective.

In testing this hypothesis, I devised a co-culture system that would enable me to replicate the *in-vivo* environment as closely as possible (Fig. 11). A layer of NSCs were propagated and allowed to spontaneously differentiate for 7 days. As shown in the previous chapter and in other studies (Gritti Angela, et al. 2001; Reynolds and Weiss 1992; Miller and Gauthier-Fisher 2009), spontaneously differentiating NSCs differentiate

into a heterogeneous population of cells that are of different neural lineages and at different stages of differentiation. GFP tagged NSCs were then co-cultured onto this heterogeneous “differentiated feeder layer” of cells. As the underlying layer of cells are alive, the GFP-NSCs would be exposed to a dynamic environment created by neural cells of different lineages and stages of development; they would thus be exposed to a myriad to biochemical signals, ECM molecules, direct cell-cell interactions and invariably, there would be physical and mechanical forces acting on the cells. As such, to some extent, the underlying layer of cells would replicate the *in-vivo* environment.

The GFP tagged NSCs were characterized between 12 hrs and 7 days in terms of morphology, the protein expression of NSC markers, cell cycle markers & neuronal markers, and functional criteria in terms of electrophysiology. The control condition was to seed GFP-NSCs in the absence of a predifferentiated feeder layer; GFP cells were seeded onto laminin coated cell culture ware, as in the standard cell culture condition.

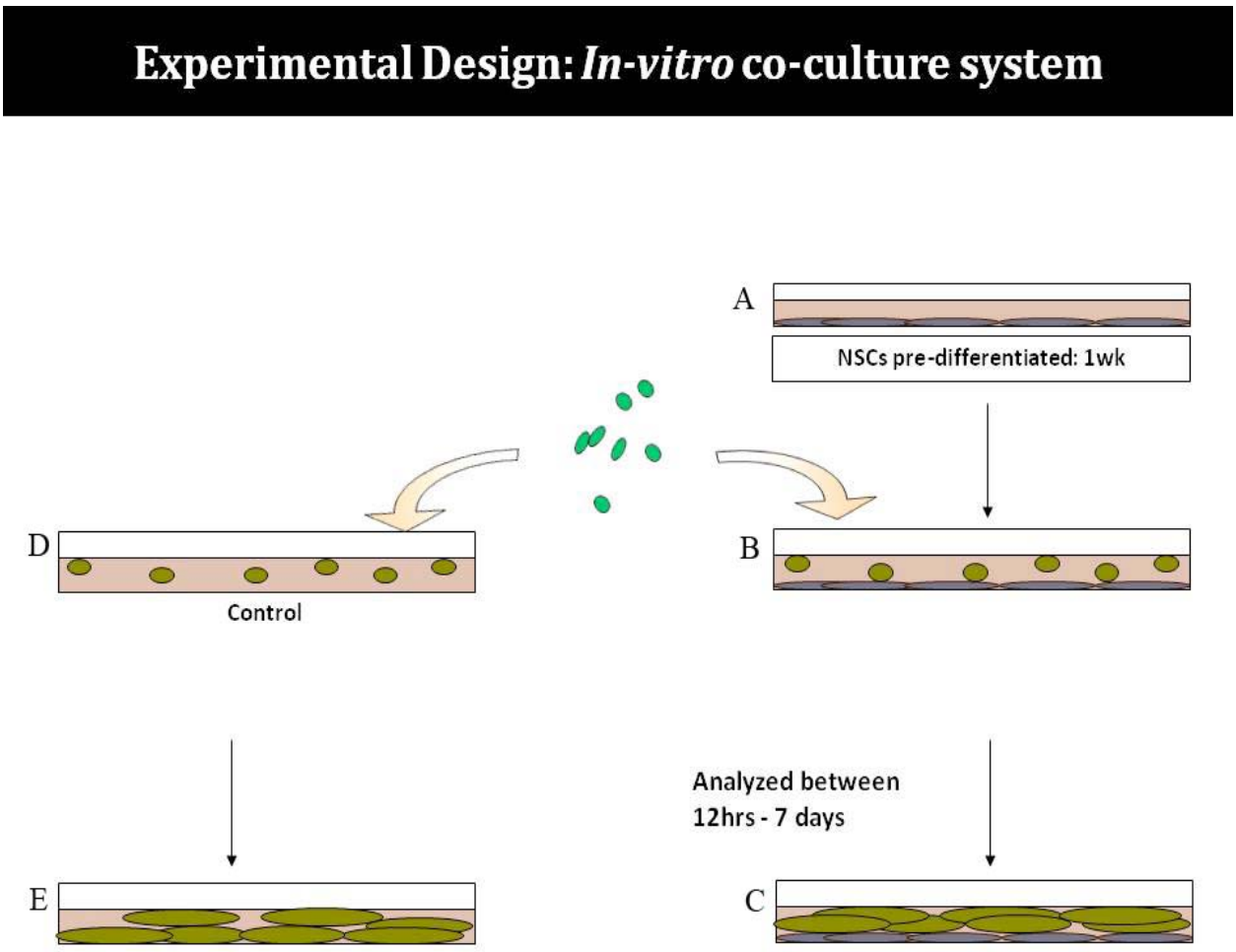


**Figure 11: Overview of the experimental design of the co-culture system.** (**A**) NSCs were seeded and differentiated for 7 days. (**B**) After 7 days of pre-differentiation, GFP-labeled NSCs were co-cultured onto the pre-differentiated layer of cells. (**C**) GFP-NSCs were analyzed at different time points of co-culture (between 12hrs and 7 days) for morphological characteristics, the expression of NSC and neuronal markers. (**D**) In the control condition, GFP-NSCs were seeded in the standard cell culture condition. (**E**) Control cells were analyzed as in condition (**C**).

## 4.2 Results

### 4.2.1 GFP-NSCs rapidly exhibit a neuronal morphology

As NSCs undergo neuronal differentiation, their morphology changes. Protrusions of neurites, which later become axons and dendrites as differentiation proceeds, appear to sprout from the cell body (Da Silva and Dotti 2002); over time, these extensions become longer and more complex with multiple sub branches. Thus, differentiation was described by morphology, in terms of the length of the neurite outgrowth and the number of neurite branches on each cell.

When fresh NSCs were incubated with a predifferentiated NSC layer, they exhibited a neuron like morphology within 12hrs of co-culture (Fig. 12B). This is not seen in the control (non co-cultured) (Fig. 12A). After 1 day of differentiation, the cells in the control condition continue to exhibit the morphology of NSCs (Fig. 12C) while the co-cultured cells had more elongated neuronal morphologies (Fig. 12D). At day 5, some cells in the control condition begin to exhibit neurite like extensions, but this was insignificant when compared to the co-cultured cells. The differentiation was then quantified in terms of the length of neurite-like extensions and the number of branches per cell. I observed significant differences in the neurite length and the number of branches per cell in the co-culture condition as compared to control conditions. While the cells in the control condition seem to retain their NSC morphology at 12hrs and 1 day of differentiation, the co-cultured cells had an 8 fold increase in terms of neurite length after 12 hrs, and a 10 fold increase after 24hrs (Fig. 12G) ($p<0.0001$). A similar trend was observed when the cells were compared in terms of their neurite branching. After 12 hrs, there was an 11 fold increase in branching number ($p<0.0001$) (Fig. 12H).

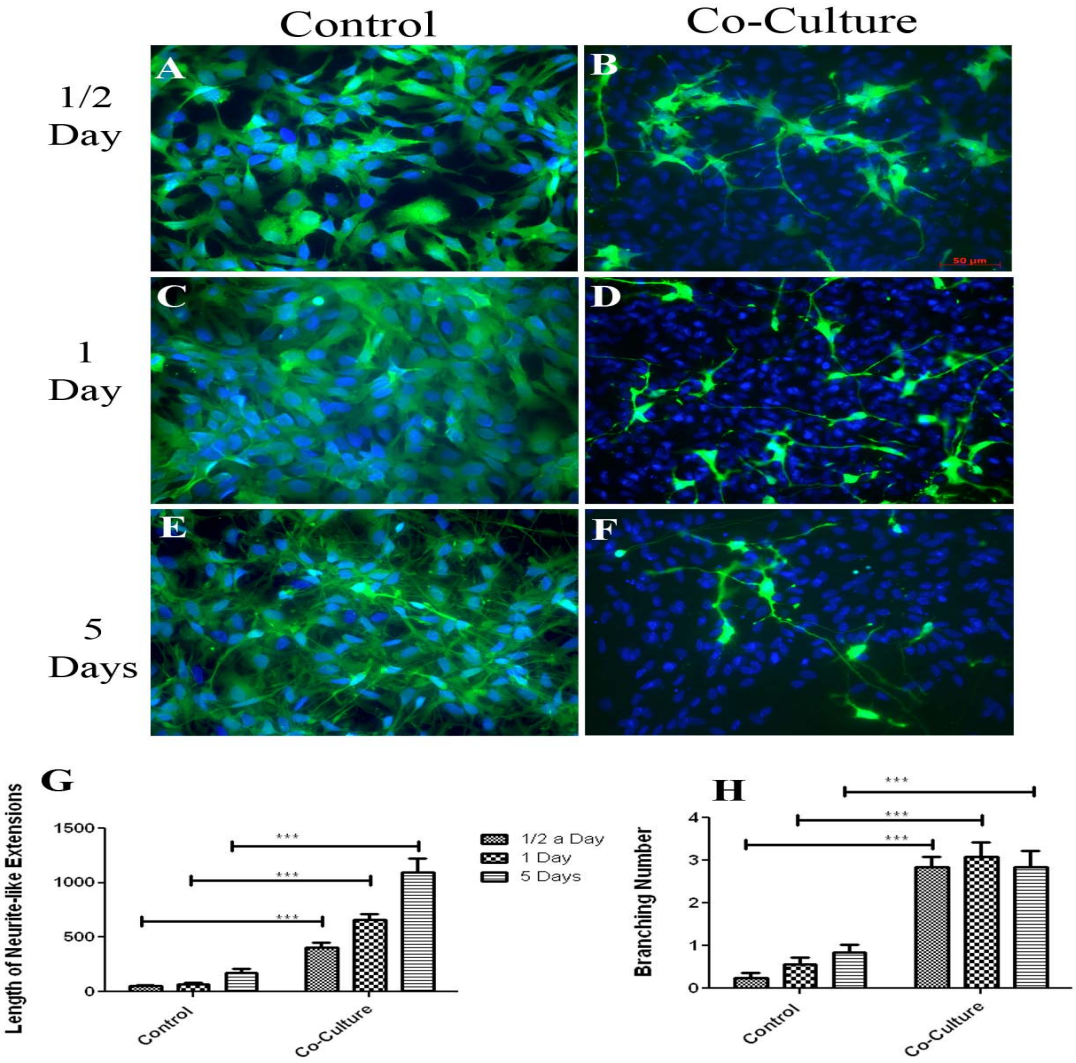


**Figure 12: GFP-NSCs co-cultured on a feeder layer of differentiated cells exhibited neuronal morphology within 12 hrs of culture.** (**A**) After 12 hrs of culture, control cells retain their NSC morphology without showing any neurite outgrowth. (**B**) In the co-culture condition, after 12hrs, GFP cells exhibited elongated neurites. (**C**) After 24hrs of culture, cells in the control condition continued to exhibit NSC morphology. (**D**) However, co-cultured GFP-NSCs exhibited longer neurites as compared to control. (**E**) After 5 days of culture, some control cells exhibited neuronal morphology with elongated neurites outgrowths (**F**) Co-cultured NSCs continued to exhibit neuronal morphology after 5 days. (**G**) Significant differences in neurite length were observed between the control and the co-cultured cells. After 5 days, the length of neurites was more than 10 fold longer as compared to control. (**H**) Analysis of the number of neurite branches. A significant 6 fold increase in branchings per cell was seen in the co-cultured cells when compared to the control. Scale bar = 50µm. ***, $p < 0.0001$.

Taken together, co-cultured NSCs showed neuron like morphology with extended neurite outgrowths and neurite branchings much faster than the control condition suggesting an accelerated neuronal differentiation of NSCs when exposed to predifferentiated cells.

### 4.2.2 GFP-NSCs rapidly down regulate NSC markers: Nestin & SOX2

Having shown that co-cultured NSCs exhibited faster neurite outgrowth, an increase in total neurite length, and an increase in the number of branching, I moved on to further investigate if NSCs differentiated faster. I assayed the down-regulation of stem cell markers such as Nestin and SOX 2 in the co-culture conditions as compared to control by immunocytochemistry at 1 day and 5 days of differentiation. Figure 13 (A-C) shows the GFP cells in the control condition after 24hrs in culture. 100% +/- 1.4 of the GFP cells still expressed Nestin after 24h of differentiation and after 5 days of differentiation still most of the cells expressed Nestin 91% +/- 5.1 (Fig. 13G). In the co-culture condition, however, within 24hrs, most GFP cells did not co-label with Nestin with only 24.4%+/- 13.6 of cells expressing Nestin. Therefore, an approximately 5 fold down-regulation was seen in Nestin within 24hrs of co-culture. This number stays relatively consistent at 5 days with 23.9% of cells continuing to express Nestin.

To further validate this observation, the expression of another NSC marker, SOX2 was analyzed. Again, as seen at 5 days, 96.9% +/- 2.1 of the cells in the control condition express SOX 2 (Fig. 14 A - C). In the co-culture, only 59.9% +/- 13 of the GFP cells express of SOX2 after 24hrs of culture and only 44.5% +/-15.2 of the GFP cell express SOX 2 after 5 days (Fig. 14 D-F). Thus, in the co-culture condition, a 50% down

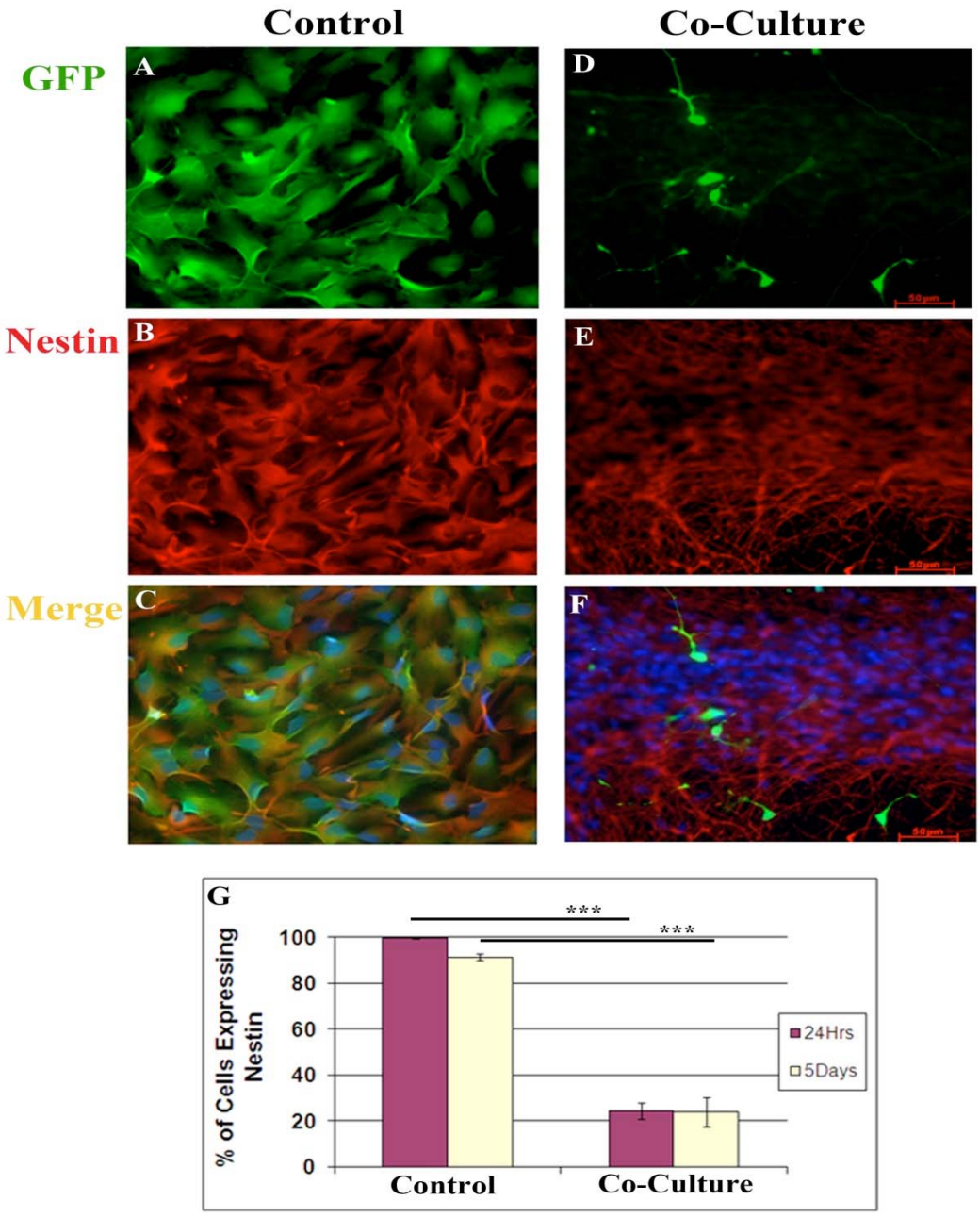


**Figure 13: Co-cultured NSCs rapidly down-regulate the NSC marker Nestin.** (**A-C**) In the control condition all GFP cells continued to exhibit the morphology of NSCs. Also, most cells expressed Nestin. (**D**) In the co-culture condition, GFP cells exhibit a different (more neuronal) morphology. (**E, F**) Co-labeling between Nestin and GFP is not observed. Nestin is mostly observed in the underlying feeder cells. (**G**) Quantification of Nestin expression showed that significant differences were observed between the 24hrs co-cultured cells and the control; there is an approximately 5 fold down-regulation of the number of GFP-NSCs that express Nestin. This remains consistent at 5 days. 91% of cells in the control condition continue to express Nestin. Scale bar = 50µm. ***, $p < 0.0001$

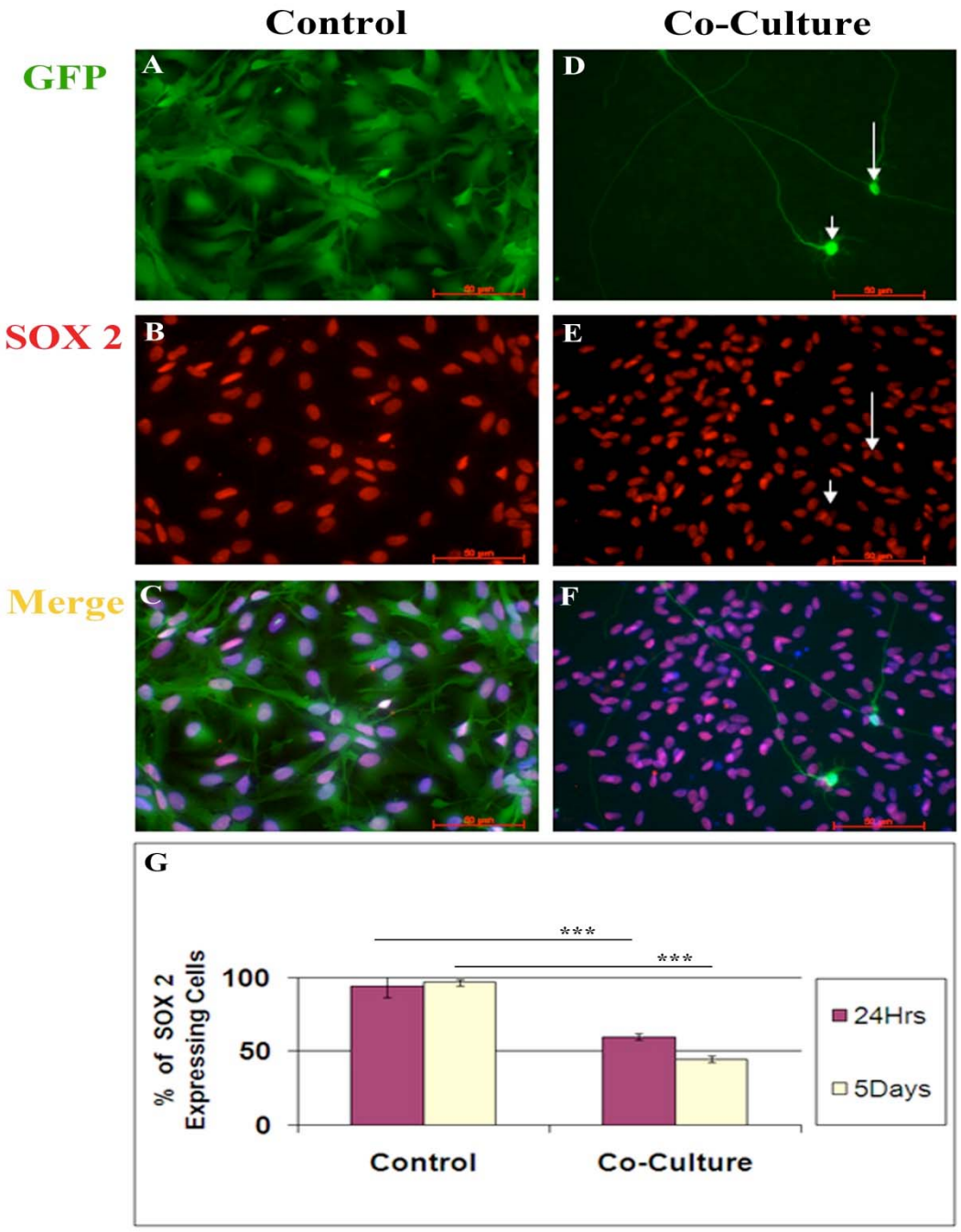


**Figure 14: Co-cultured NSCs rapidly down-regulate the NSC marker SOX2. (A-C)** In the control condition, all GFP cells continued to exhibit the morphology of NSCs and all cells expressed SOX 2. (**D-F**) In the co-culture condition, GFP cells exhibit a very different (more neuronal) morphology, with elongated neurite outgrowth. No co-labeling between SOX 2 and GFP was observed (white arrows). (**G**) Quantification of SOX 2 expression shows that significant differences were observed between the 24hrs co-cultured cells and the control. There was an approximately 2 fold down-regulation of the number of GFP-NSCs that express SOX2. This remains consistent at 5 days. Approximately 45% of cells in the control condition continue to express SOX 2. Scale bar = 50µm. ***, $p<0.0001$

regulation was observed in SOX 2 expression , within 24 of culture, which is not seen in the control condition even after 5 days (Fig. 14G).

Thus taken together, the co-culture condition rapidly down regulates NSC markers, Nestin and SOX 2, which in the control condition remained relatively highly expressed. The continued presence of NSC markers at 5 days of co-culture differentiation could indicate that some cells are refractory to differentiation, thus maintaining their 'stemness'.

### 4.2.3 Co-cultured GFP-NSCs rapidly up regulate neuronal markers

Having observed that co-cultured NSCs down regulate NSC markers, I further investigated the differentiation of NSCs by studying the regulation of neuronal markers Tuj1, MAP2 and TH. GFP-NSCs were co-cultured onto a predifferentiated layer of neural cells and analyzed after 5 days of co-culture. Co-cultured GFP-NSCs were compared with NSCs differentiated in the control condition. Looking at Tuj 1 expression, as seen by white arrows, a significant percentage of GFP cells co-labeled with Tuj 1, within 5 days of co-culture (Fig. 15A-C). When this observation was quantified, 35.7% +/- 11.9 of the co-cultured GFP cells expressed Tuj1 while only 16.2% +/- 2.5 of the control cells expressed the immature neuronal marker (Fig. 15D). On looking at mature neuronal marker, MAP 2 (Fig. 15E-G), 27.2% +/- 7.2 of the GFP cells co-labeled with MAP 2 staining, while only 0.51% +/- 0.35 of the control cells expressed MAP 2. On looking at the expression of the Catecholamine (DA) phenotypic neuronal marker, TH (Fig. 15 I-L), 6.4% +/- 4.5 of the co-cultured GFP cells expressed TH, while only 1.3% +/-0.7 of the control cells expressed the marker. Thus, the co-culture condition enhanced the expression of neuronal markers as there was more than a 2fold increase in the number

of cells expressing Tuj 1, ~30 fold increase in the number of cells expressing MAP 2 and ~4 fold increase in the number of cells expressing TH. Furthermore, this enhanced up-regulation of neuronal markers occur at a more accelerated pace as compared to the control.

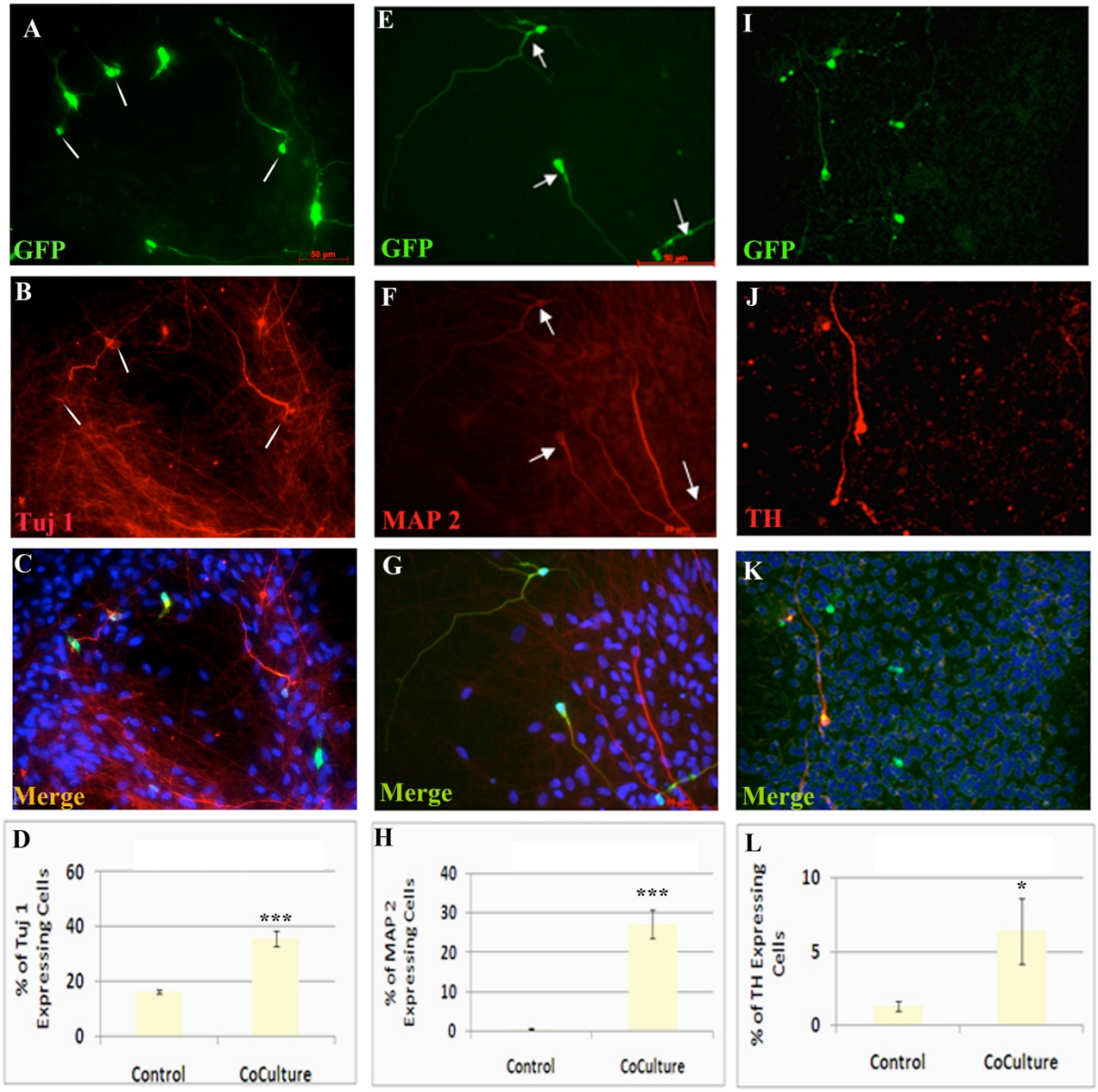


**Figure 15: Co-cultured NSCs rapidly up-regulate the neuronal markers Tuj 1 (B, C), MAP 2 (F, G) and TH (J, K). (A-C)** GFP-NSCs exhibit a neuronal morphology at 5 days of differentiation). White arrows represent cells that co-label with GFP and Tuj 1 (β-Tubulin-III). (**D**) The quantification showed significant differences in Tuj 1 expression between the control and the co-cultured cells. (**E–G**) show the co-labeling of MAP 2 and GFP. The white arrows point to GFP cells that also labeled with MAP 2. (**H**) Quantification of of MAP 2 expression shows more than 20 fold increase in the number of MAP expressing neurites. (**I-K**) GFP-NSCs colabeled with the neurotransmitter phenotype marker TH. (**L**) A significant 5 fold up-regulation of TH is observed in the co-culture conditions. *, $p< 0.05$; ***, $p<0.0001$. Scale bar = 50µm

### 4.2.4 Temporal profile of rapid differentiation

I showed above that co-cultured NSCs undergo accelerated and enhanced differentiation. In order to understand the dynamics of this accelerated and enhanced differentiation in more detail, I investigated the temporal aspect of differentiation of these cells between 0 days and 7 days of co-culture differentiation. As in the previous chapter, the cultures were assayed for Nestin, Ki 67, β-III Tubulin and TH expression via immunocytochemistry (Fig. 16). Nestin and Ki 67 (Fig. 16 A & B) would assay the maintenance of 'stemness' while β-III Tubulin and TH (Fig 16 C & D) serve as neuronal differentiation markers.

Looking at Nestin (Fig. 16A), I observed that within 24hrs its protein expression 4-5 folds down regulated and it stays at this level throughout the duration of 7 days. This is unlike the standard culture method (see Fig. 6A) where Nestin is down regulated to 80% after 4 days of differentiation and remains at this level even at 7 days of differentiation.

As with Nestin, the proliferation marker Ki 67 was also rapidly down-regulated (Fig 16B). Within 1 day of differentiation, Ki 67 expressing cells dropped to 41.8%+/- 6.5 and in the further course of differentiation Ki 67 is virtually undetectable in cells, . Therefore, compared to the standard differentiation protocol (see Fig. 6B), both Nestin and Ki67 is more rapidly down-regulated in the co-culture system. Thus, the data suggests that co-cultured NSCs lose their 'stemness' more quickly and more efficiently as a consequence of the presence of the differentiated feeder layer. In line with the rapid down-regulation of stemness markers, it was observed that β-III-Tubulin was expressed in about 45%+/- 5.3 of the GFP-NSCs at day 4 of co-culture.

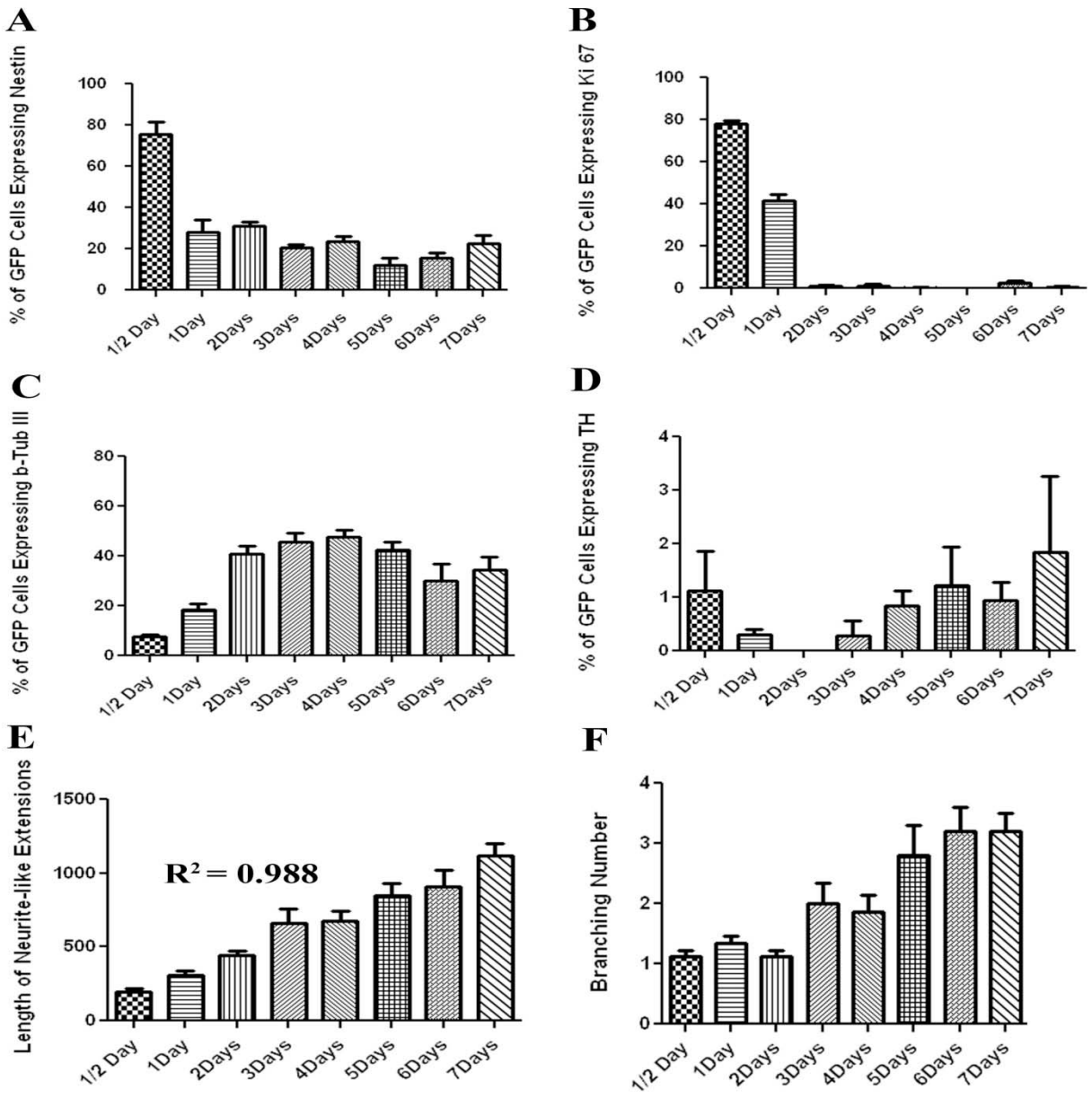


**Figure 16: 7 days characterization co-cultured GFP-NSCs.** (**A**) Quantification of Nestin expression. Within 24hrs, Nestin expression is down-regulated to about 20% and remains at this level of expression. (**B**) Quantification of Ki 67- expresison. Within 24hrs, Ki 67 expression is down-regulated to about 40% and by 2 days of co-culture, Ki 67 expression is virtually absent. The change in expression levels of Nestin and Ki 67 may suggest that the GFP cells rapidly lose their 'stemness'. (**C, D**) shows the regulation of neuronal markers, β-III-Tubulin and TH. Within only 4 days about 45% of cells express neuronal marker, β-III-tubulin. (**D**) Though some regulation is seen with TH, it is not significantly regulated ($p > 0.05$). Changes in (**A**), (**B**) & (**C**) are significant ($p < 0.0001$). (**E, F**) characterizes the morphology of the co-cultured cells. With a $R^2$ value of 0.988, the neurite extensions seem to increase proportionately over time (**E**). Similarly, the branching complexity increases, but seems to plateau after 5 days (**F**).

However, after day 4, the expression begins to decline to about 33%+/-10.5 (Fig. 16C). This value is ~ 5 times higher than that of NSCs cultured via the standard culture method as only 6.69%+/-2.75 of cells express β-III Tubulin (See Fig. 8A). Furthermore even after 7 days of differentiation, more than 11% of the cells differentiated in the standard culture method did not express β-III-Tubulin (See Fig. 8A), indicating the enhanced differentiation of the co-culture method. No significant difference is seen in the expression of TH over 7 days.

On analyzing the morphology of the co-cultured NSCs, a steady linear increase is seen in terms of the neurite length (Fig 16E) ($R^2$ = 0.988). As noted above (See Fig.12) these lengths are significantly more elongated than the neurite length of control cells. It should be noted, however, that most cells have extensions that go beyond the frame of view by the $6^{th}$ day of differentiation. Thus, the measurements shown here indicate the neurite length within the field of view. A similar trend is seen with the branching complexity, though it plateaus after day 5 (Fig 16F).

### 4.2.5 Electrophysiological characterization of co-cultured GFP-NSCs

As the differentiation of co-cultured GFP-NSCs is accelerated and enhanced, I wanted to study if cells rapidly matured electrophysiologically by using Patch Clamp analysis. In particular, I wanted to learn if co-cultured NSCs were able to elicit action-potential. Due to technical difficulties that arise due to the dense nature of the co-culture system, we were unable to directly Patch and analyze the co-cultured NSCs. GFP-NSCs were thus separated from the co-culture condition after 24hrs of co-culture via FACS sorting and seeded onto cover slips for 24hrs before Patch Clamping analysis. The control

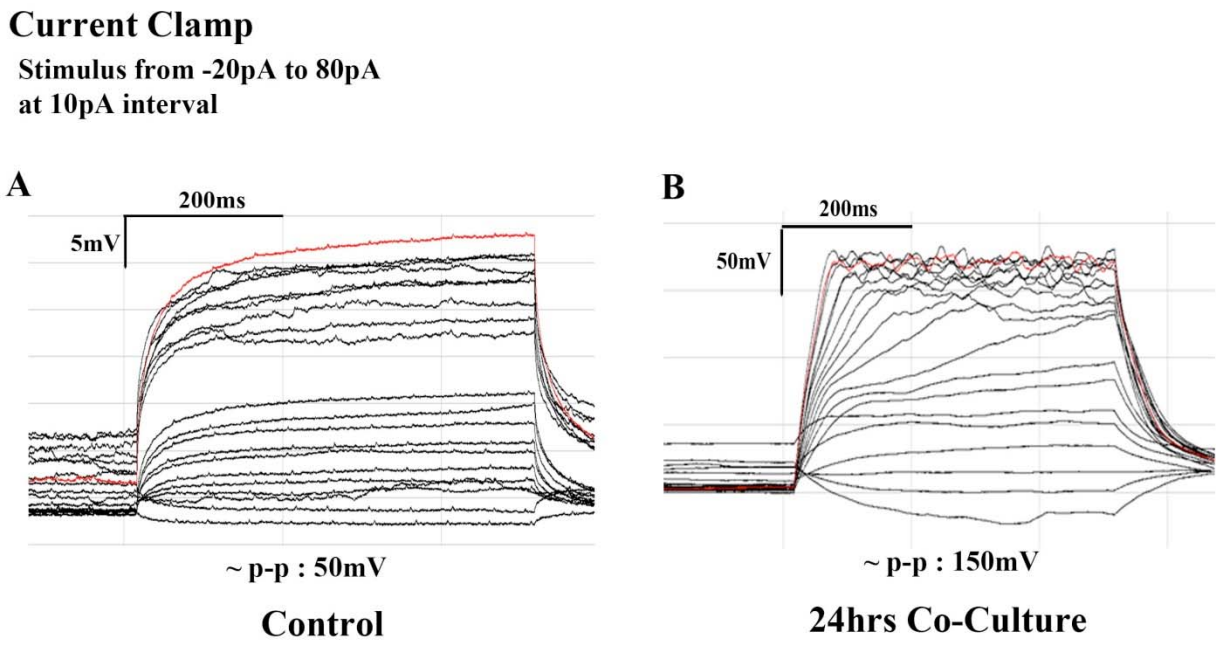


**Figure 17: Electrophysiological analysis of co-cultured NSCs.** Using the whole-cells current clamp configuration, cells were stimulated between -20pA and 80pA. (**A, B**) shows graphs representing the electrophysiological properties of cells under the current clamp configuration. The objective of this current clamp is to understand if these cells elicit action-potential. (**A**) Represents cells cultured in control conditions for 24hrs and (**B**) represents cells cultured in co-culture for 24hrs. Though the peak – peak amplitude of the voltage response in B was approximately 3 times higher than A, no action-potential was observed in both conditions, thus indicating that that the cells probed were not electrophysiologically mature neurons.

consisted of cells differentiated via the standard culture method for 24hrs, trypsinized and re-seeded onto cover-slips for 24hrs prior to Patch Clamping analysis. At a holding potential of -70mV, step currents were injected between -20pA and 80pA at intervals of 10pA. Results indicate that though differences were observed the voltage amplitude between the co-cultured cells and control cells, neither of them elicit an action potential (Fig. 17B).

### 4.3 Summary

I hypothesized that if the extrinsic cues present in the *in-vivo* environment were replicated in a NSC culture system, neuronal differentiation of NSCs would be more efficient and effective. In testing this hypothesis, I devised a co-culture system where NSCs were seeded onto a predifferentiated layer of neural cell. Results indicate that the hypothesis is validated as co-cultured NSCs undergo accelerated and enhanced differentiation. They rapidly exhibit neuronal morphology; they rapidly down regulate NSC markers and up-regulate neuronal markers. The effect is accelerated as these effects are seen within 12-24 hrs and they are enhanced because some of these results, such as the 5-fold down regulation of Nestin, are not seen in NSCs differentiating via the standard culture method even after longer durations. We thus move on to better understand this phenomenon of accelerated and enhanced differentiation.

**Chapter 5:**

# The effect of accelerated and enhanced differentiation requires a live predifferentiated layer of neural cells

## Chapter 5:
## The effect of accelerated and enhanced differentiation requires a live predifferentiated layer of neural cells

### 5.1 Introduction

The experiments in this chapter act as controls. From the previous chapter, it is known that co-cultured GFP-NSCs down regulate NSC markers, exhibit neuronal morphology and express neuronal marker. We attempted to better understand what the cause for this effect could be. Till this point, the condition that stimulates this phenomenon is a feeder layer of cells differentiating for 7 days. The question remains whether differentiated feeder cells were required or merely a co-culture system with any cell type (differentiated or not) was sufficient for this effect. This question is addressed in this chapter.

In 2008, it was shown that co-culturing human Embryonic Stem Cells (hESCs) with a fixed layer of PA6 cells produced the same number of β-III-tubulin positive neurons as co-culturing hESCs on live PA6 cells (Vazin, et al. 2008). I investigated if this phenomenon of accelerated and enhanced differentiation was similar to that shown by Vazin and colleague, whereby a fixed layer of cells produced the same effect.

Thirdly, in order to understand if this phenomenon was specific to the particular cell-line used as the feeder layer, its effect was compared by the use of another neuronal cell line as the feeder layer.

Thus, in the following chapter I investigate whether differentiated cells were required to elicit accelerated differentiation, whether the feeder cells needed to be living cells and if this phenomenon was limited to the cell line used so far or whether it was a more general phenomenon.

## 5.2 Results

### 5.2.1 Feeder layer cells require pre-differentiation for inducing accelerated and enhanced differentiation of NSCs

NSCs were seeded and propagated as described. At different time points of pre differentiation (0, 1, 3, 5, 7 days), fresh GFP-tagged NSCs were seeded onto the layer of pre-differentiated cells and co-cultured for another 24hrs. The undifferentiated (0 days differentiated) feeder act as the control and I first analyzed neurite-like extensions and branching points per cell as a function of the time of predifferentiation.

It was observed that GFP-NSCs seeded onto 1, 3, 5 and 7 day predifferentiated feeder cell layer showed extensions that were significantly longer than GFP-NSCs seeded on an undifferentiated feeder (control) ($p<0.001$) (Fig.18A-F). An approximately 3-fold increase was observed in the neurite extensions of GFP-NSCs co-cultured on a 1 day predifferentiated feeder as compared the control; this value remained consistent with the neurite extensions on GFP-NSCs seeded on a 7 day pre-differentiated layer (Fig. 18F).

Similarly, in terms of the number of branchings on cells, NSCs seeded onto a 3, 5 and 7 days predifferentiated feeder cell layer had neurites that were about 4 times more branched than those on the control ($p<0.0001$) (Fig. 18A-E & G); the number of branches on GFP-NSCs co-cultured on the control and the one day predifferentiated feeder layer was significantly less branched than those co-cultured on a 5 and 7 day pre-differentiated layer.

Thus taken together, it can be said that the predifferentiation of the underlying feeder cells played a role in the rapid exhibition of neuronal morphology observed in the

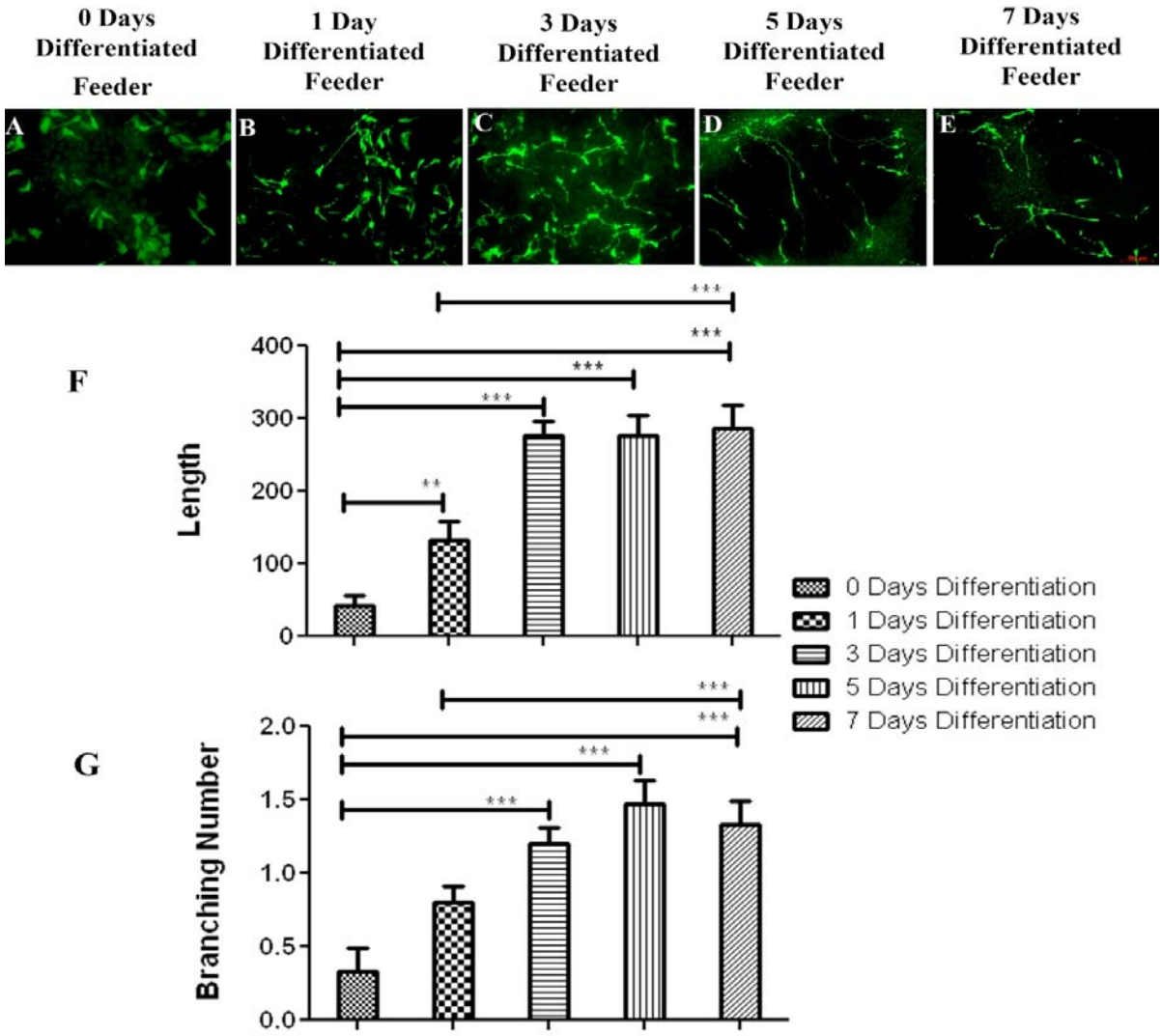


**Figure 18: Quantification of neurite length and number of branches as a function of predifferentiation of feeder cells.** (**A - E**) GFP-NSCs co-cultured for 24 hrs on feeder layers at different time-points of differentiation. (**A**) NSCs on undifferentiated feeder layer retain an undifferentiated morphology. (**B**) NSCs on 1 day predifferentiated feeder cells have cells of mixed morphology – some retain a globular NSC morphology while others have a more elongated neuronal morphology. (**C**) NSCs on 3 days predifferentiated feeder have majority of the cells with elongated neuronal morphology, (**D, E**) NSCs on 5 days predifferentiated feeder and NSCs on 7 days predifferentiated feeder primarily have elongated neuronal morphology. (**F**) Quantification of neurite length of co-cultured NSCs on feeders cell predifferentiated for different length of time. As observed in (**A-E**), a longer period of predifferentiation of the feeder layer, influences the length of neurites. Neurites of cells grown on all differentiated feeders are significantly longer than cells co-cultured on the undifferentiated feeders. (G)GFP cells on the longer predifferentiated feeders exhibit more complex neurite branching. Thus, there is a positive correlation between the length of feeder differentiation and the degree of differentiation of GFP-NSCs. Scale bar = 100um. **, $p < 0.01$; ***, $p < 0.0001$

co-cultured NSC. From my data, 3 - 5 days of predifferentiation was sufficient in eliciting this effect.

Knowing that the underlying layer of cells induced morphological differences on the co-cultured GFP-NSCs I next studied Nestin, Ki 67, β-III-tubulin and TH regulation on the level of immunocytochemistry. All GFP cells seeded in the control expressed Nestin 24hrs after co-culture (Fig. 19 A-C & J). However, in correlation with the differentiation of the feeder layer, Nestin was down regulated; from 3 days of feeder predifferentiation, Nestin is significantly lower than the control ($p<0.0001$) (Fig. 19J).

Though ~80% of the GFP cells in the control condition express Ki 67 (Fig. 20), there was a significant 2 fold down regulation of Ki on the 1 day predifferentiated feeder ($p<0.001$). Ki 67 expression of GFP cells seeded on the 3 days predifferentiated feeder is further reduced to 8.6% +/-5 which remains consistent for cells seeded on the 5 and 7 day feeders.

GFP cells on the control feeder do not express β-III-Tubulin (Fig. 21. A-C). However, as noticed by the thin white arrows (Fig. 21 D - I), the expression of β-III-Tubulin on GFP cells was significantly increased to 8.2% +/-5.3 and 9.6% +/-5.7 on 3 days predifferentiated and 5 days predifferentiated feeders respectively (Fig. 21J). The expression of TH (Fig. 22), on the other hand was not significantly regulated. However, looking at the absolute numbers, it follows the same trend as β-III-tubulin, with an increase of expression within 3 days of feeder differentiation.

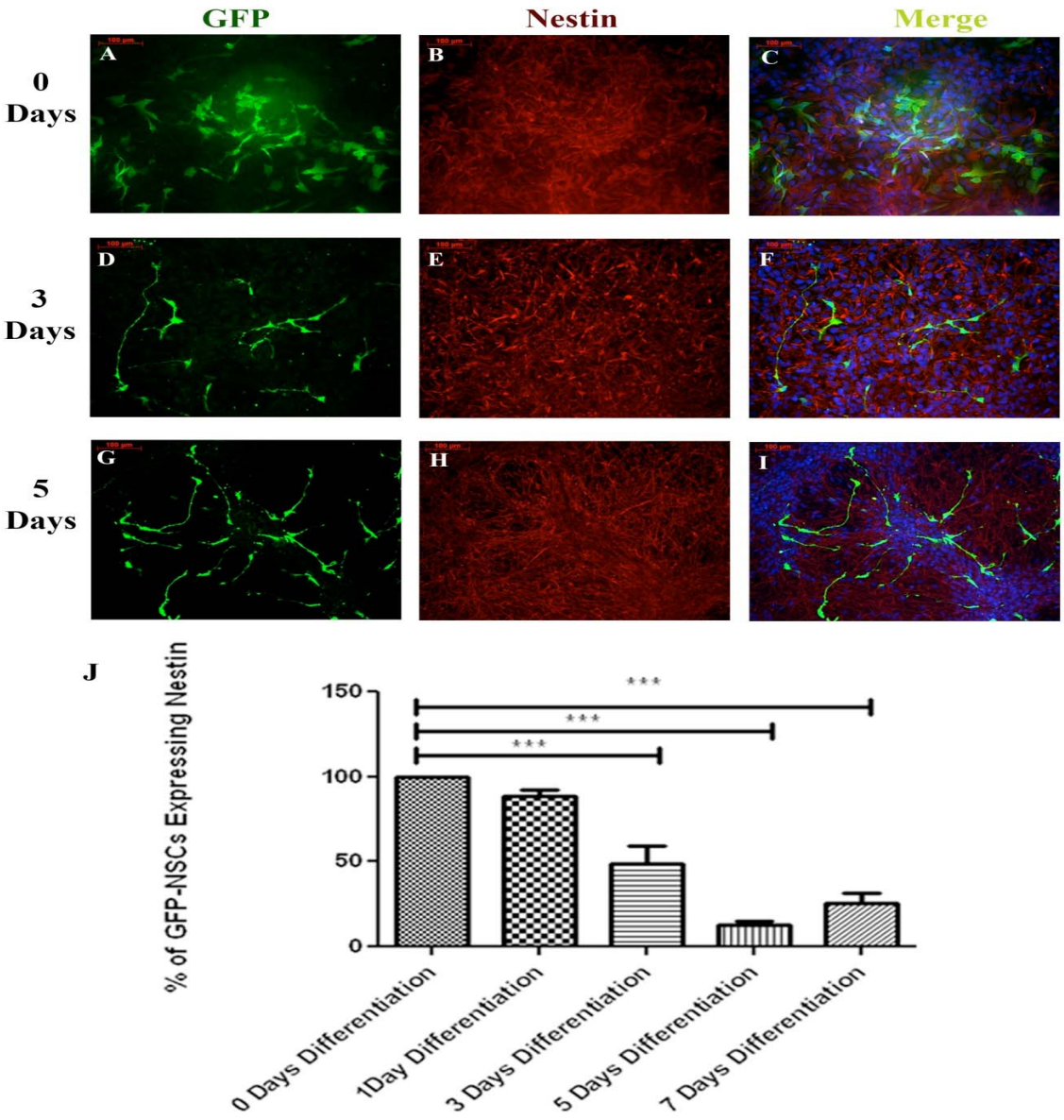


**Figure 19: The level of the NSC marker Nestin down regulation correlates with the length of predifferentiaton of the feeder cells.** (**A-C**) At 0 days of feeder predifferentiation, all GFP cells co-labeled with Nestin after 24hrs of culture. (**D-F**) At 3 days of feeder predifferentiation, GFP cell morphology had more neuronal features and few cells co-label with Nestin. **(G-I)** After 5 days of feeder predifferentiation, GFP cells have neuronal morphology and fewer cells co-label with Nestin. (**J**) The percentages of GFP-NSCs expressing Nestin are quantified for the various predifferentiation timepoints. At 3 days of predifferentiation, the percentage of GFP cells that co-labeled for Nestin significantly reduce to approximately 50%. Only 20% of GFP cells on 5 and 7 days predifferentiated feeders continued to express nestin which indicate further downregulation. Thus, the percentage of nestin positive cells are correlated negatively with the length of predifferentiation of the feeder layer cells. Scale bar=100μm. ***, $p<0.0001$.

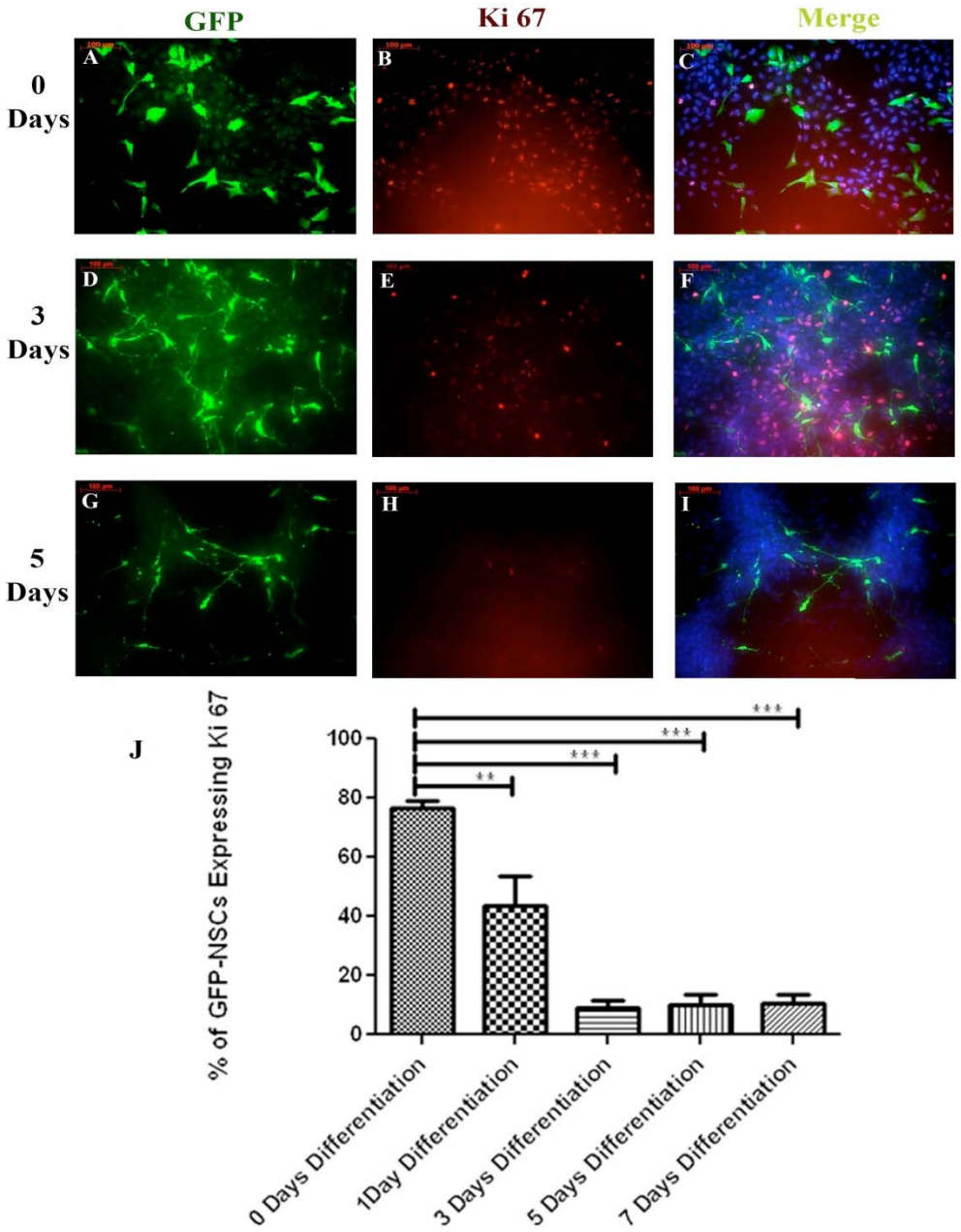


**Figure 20: The down regulation of the cell cycle marker Ki 67 correlates with the length of the predifferentiation of feeder cells.** (**A-C**) After 24hrs of co-culture on 0 day differentiated feeder, all GFP cells were active in the cell cycle. (**D-F**) On the 3 days differentiated feeder, GFP cells that co-labeled with Ki 67 was significantly reduced. Most of the Ki 67 staining in are from the feeder layer. (**G-I**) Similarly, at 5 days, most GFP cells do not express Ki 67. Less Ki 67 staining is noticed because most of the feeder cells also exit the cell-cycle. (**J**) Quantification of the percentages of GFP-NSCs expressing Ki 67 shows that at 1 day of predifferentiation, the GFP cells that co-label for Ki 67 significantly reduces to about 50%. GFP cells on 3, 5 and 7 days predifferentiated feeders further down-regulate Ki 67 to about 10% ($p<0.0001$). Thus, the percentage of GFP cells that are active in the cell-cycle correlates negatively with the length of predifferentiation of the feeder layer cells. Scale bar=100μm. **, $p<0.01$; ***, $p<0.0001$.

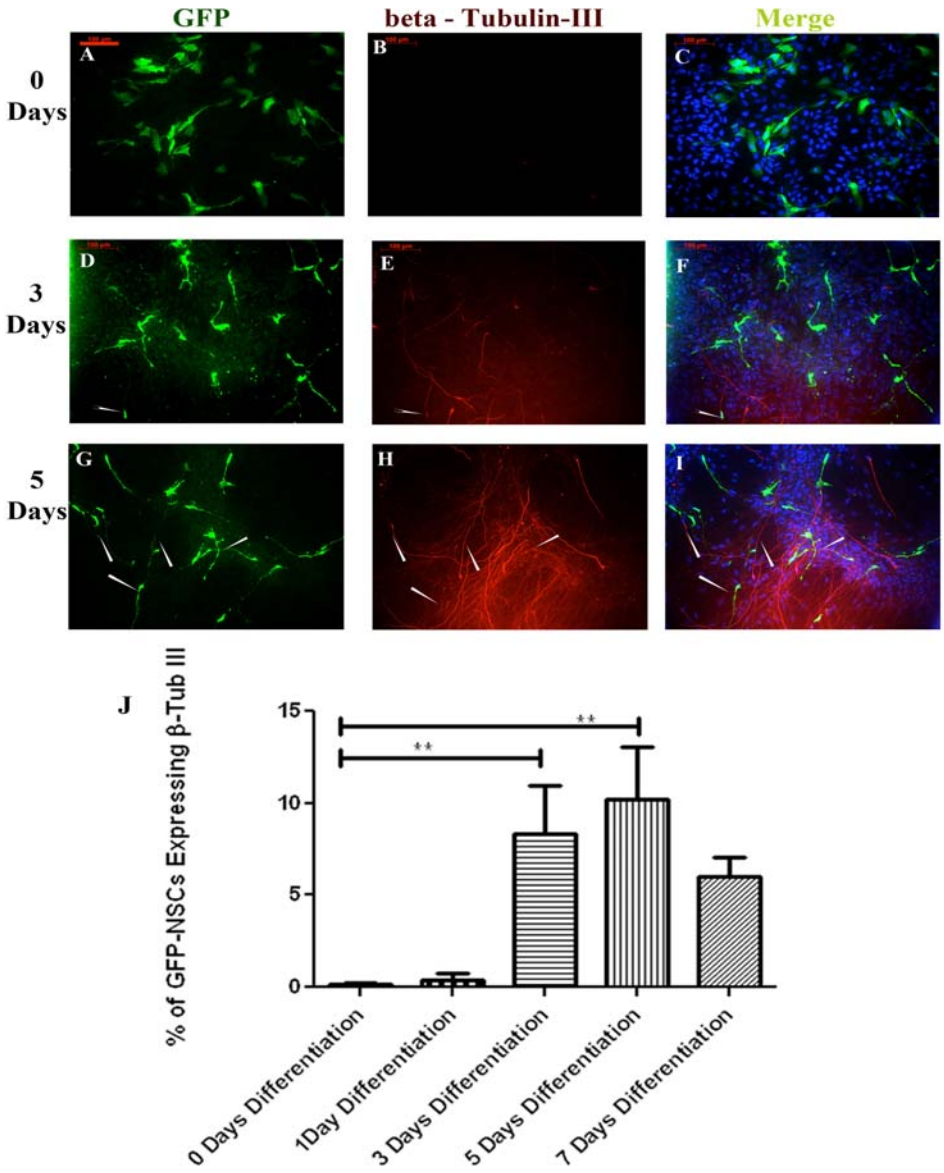


**Figure 21: The up regulation of neuronal marker, β-III-Tubulin correlates with the length of the predifferentiation of feeder cells. (A-C)** On day 0 feeder, after 24hrs of co-culture, no β-III-Tubulin expression was observed on GFP cells. (**B-F**) On the 3 days predifferentiated feeder, the GFP cells that expressed β-III-Tubulin significantly increases (white arrows). **(G-I)** GFP cells on the 5 days predifferentiated feeder further up-regulates β-III-Tubulin (white arrows). **(J)** Quantification of the percentages of GFP-NSCs expressing β-III-Tubulin shows that an insignificant number of GFP cells co-label with β-III-Tubulin when seeded on day 0 and day 1 feeders. The expression was significaltly increased to about 7% and 12%, when seeded of 3 days and 5 days feeders respectively. Thus, from β-III-Tubulin expression, it can be said that neuronal differentiation is correlated with the length of predifferentiation of the feeder layer cells. Scale bar=100μm **, $p<0.001$.

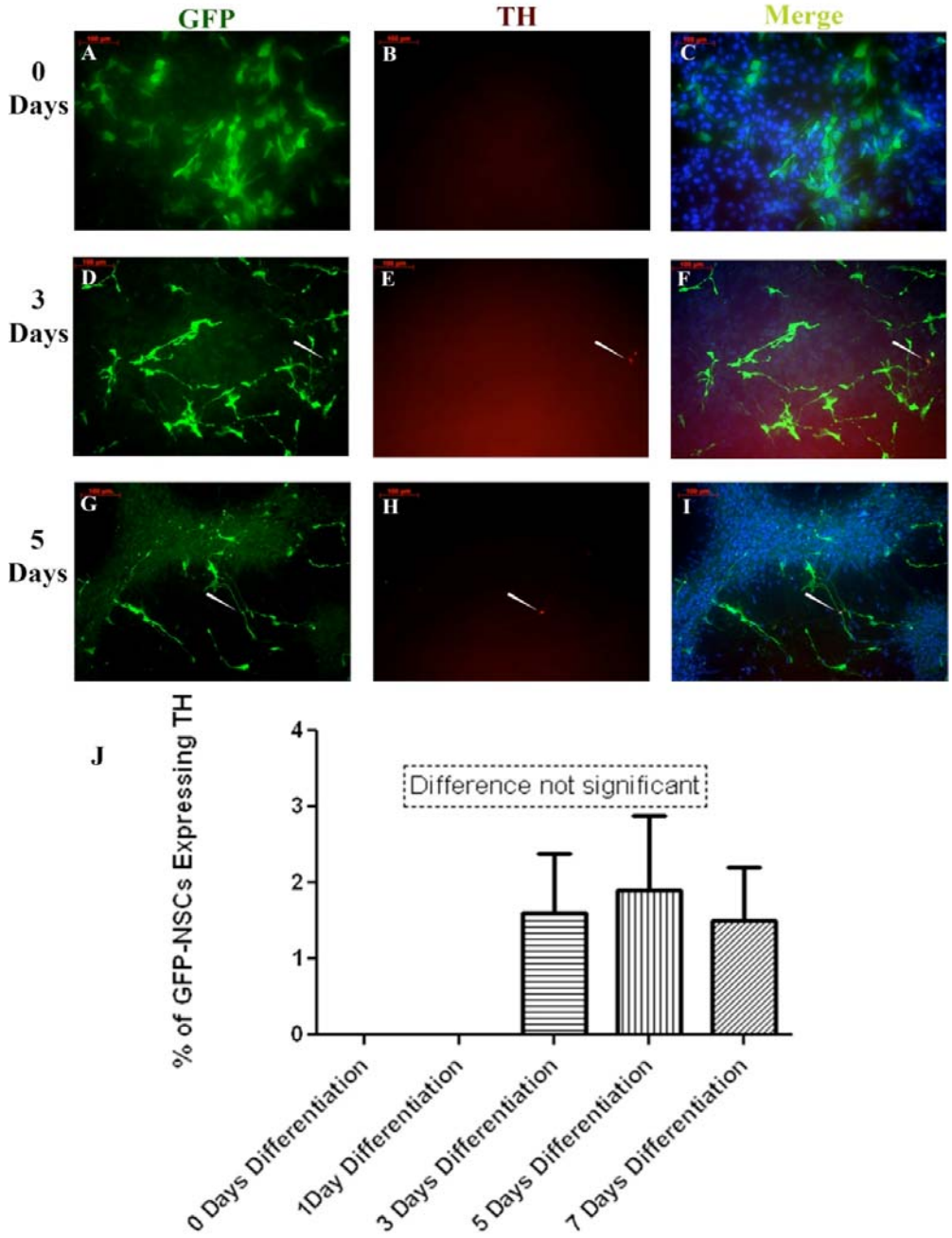


**Figure 22: TH expression does not show signficant changes when co-cultured on predifferentiated feeder cells. (A-C)** On day 0 feeder, after 24hrs of co-culture, no TH expression was observed on GFP cells. (**B-F**) On the 3 days predifferentiated feeder, the GFP cells that expressed TH increased (white arrows). **(G-I)** Though GFP cells exhibit more neuronal morphology, on the 5 days predifferentiated feeder, no increase of TH labeling is observed. **(J)** Quantification of the percentages of GFP-NSCs expressing TH - Though the number of cells that co-label GFP and TH seem to increase on the 3 day feeder, the data is not significant. This is probably due to the low number of cells. Scale bar=100μm.

Therefore differentiated cells are required for the effect of accelerated and enhanced differentiation.

### 5.2.2 Accelerated and enhanced differentiation of NSCs requires live cells

Having shown that the predifferentiation of the underlying layer of cells affected the differentiation of co-cultured NSCs, we probed whether the feeder layer needs to be alive to result in rapid and enhanced differentiation. Thus, 7 days predifferentiated NSCs were fixed by different methods and NSCs were co-cultured onto the layer of predifferentiated and fixed cells. The fixation methods used fall generally into two classes: organic solvents and cross-linking reagents. Organic solvents such as alcohols remove lipids and dehydrate the cells, while precipitating the proteins on the cellular architecture. Cross-linking reagents, such as paraformaldehyde (PFA) form intermolecular bridges, normally through free amino groups, thus creating a network of linked antigens. Cross-linkers preserve cell structure better than organic solvents, while solvents may better preserve the antigenicity of some cell components. As such, both methods of fixation were used; 4% PFA (Fig. 23C), 95% ethanol (EtOH) (Fig. 23D) and 100% methanol (MeOH) (Fig. 23E) were used for the fixation. Cells co-cultured on these conditions were compared with the control condition, where cells were seeded either on laminin coated tissue culture plastic (Fig. 23 A) or co-cultured on a live layer of predifferentiated cells (Fig. 23B).

After 24hrs of co-culture, the neurite-like extension and number of branches were assayed. GFP-NSCs seeded on laminin (control) (Fig. 23 A) retained their NSC morphology, while NSC co-cultured with live predifferentiated cells exhibited neuronal morphology (Fig. 23 B). GFP-NSCs co-cultured onto a layer of PFA fixed feeder layer continued to exhibit NSC morphology (Fig. 23C). EtOH and MeOH fixed feeders seems

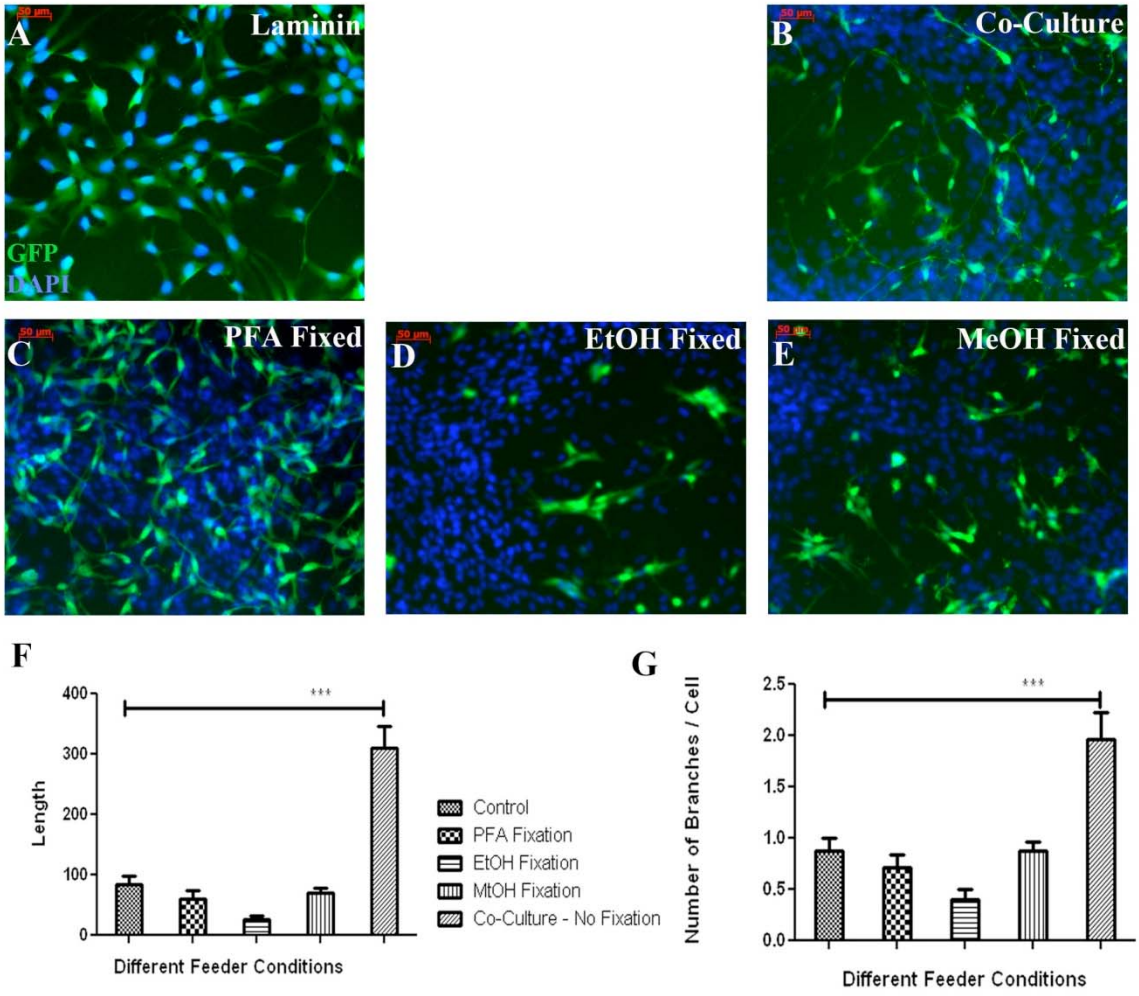


**Figure 23: Fixation inactivated pre-differentaited feeder cells do not induce enhanced and accelerated differentiation.** (**A**) GFP-NSCs in the control conditions after 24hrs of seeding. (**B**) GFP-NSCs co-cultured with a layer of pre-differentiated cells. (**C- E**) Co-culture with GFP-NSCs seeded onto a layer of fixed predifferentiated neural cells that are inactivated by (**C**) PFA fixation, (**D**) Ethanol fixation, and (**E**) Methanol fixation. (**F**) Quantification of neurite length and (**G**) number of branching points per cell. When compared with co-cultured GFP cells, significant morphological differences were observed in the GFP-NSCs seeded on the inactivated feeder layers (as in conditions C, D and E**)**. All conditions were significantly different from the co-culture with live predifferentiated cells. Scale bar=100 . ***, $p<0.0001$.

to repel the co-cultured NSCs as they tend to cluster together at regions without feeder cells (Fig 23 D&E). None of the fixed cell layers induced neuronal differentiation morphology as no significant differences were observed between the laminin control condition and fixation conditions in terms of neurite outgrowth and neurite branching (Fig. 23 F&G). Only cells co-cultured on live predifferentiating cells evoked neuronal differentiation morphology. Thus, fixed cells do not generate the effect of rapid and enhanced differentiation and as such live cells were required.

### 5.2.3 Accelerated and enhanced differentiation could be a general feature of NSCs

So far, the results I presented have been achieved with the same population of NSCs, both as feeders as well as the cell population seeded on top the predifferentiated feeders. In order to rule out that this effect is specific to this particular NSC line, we investigated another and independent NSC population as feeder layers; ReNcell-CX (CX) (Millipore, Billerica, MA) originally derived from the fetal brain was used. Unlike the cells previously used, which were derived from the midbrain region of the fetal brain, these cells were derived from the cortical region of the fetal brain. CX cells were cultured and propagated to get the appropriate cell density (80% confluency). Upon getting the appropriate cell numbers, growth factors were removed and cells were allowed to spontaneously differentiate for 7 days. At day 7 of predifferentiation, GFP-NSCs were seeded in co-culture and analyzed after 24hrs. Cells were assayed for morphological differences characterized by the neurite-like extensions and the number of branching on each cell. In the control condition, (NSCs on laminin) cells retained their NSC morphology after 24hrs of culture (Fig. 24A). In the co-culture conditions, both on the CX feeder as well as the VM feeder, GFP-NSCs exhibited neuronal morphologies (Fig.

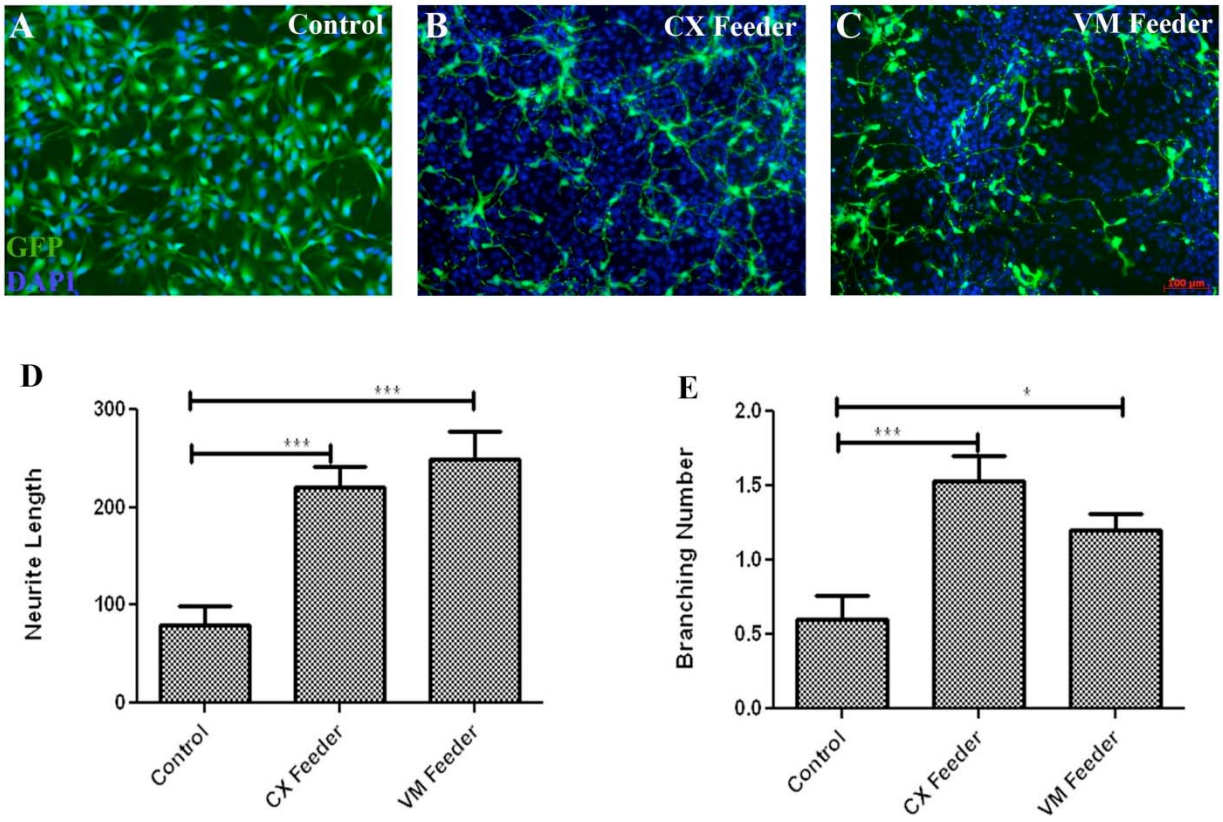


**Figure 24: Accelerated and enhanced differentiation is not specific to the ReNeuronVM cells.** (**A**) Culture of GFP-NSCs in the control condition after 24hrs of seeding. GFP cells do not exhibit neuronal morphology. (**B**) GFP-NSCs co-cultured on a pre-differentiated layer of cortical NSCs (ReNeuronCX). GFP cells exhibit neuronal morphology. (**C**) GFP-NSCs co-cultured on a pre-differentiated layer of mid-brain derived NSCs (ReNeuronVM). GFP cells also exhibit neuronal morphology. (**D, E**) Quantification of neurite length and the number of branching points. Significant morphological differences are not observed between the GFP-NSCs in **B** or **C**. However, both conditions are significantly different from the control. Scale bar=100. *, $p<0.005$. ***, $p<0.0001$.

24 B & C). In terms of the neurite-like elongations, both neuronal feeders were approximately 3 times more elongated than the control ($p<0.0001$)(Fig. 24D). Similarly, in terms of the number of branchings, more than a 2 fold increase was observed when compared with the control ($p< 0.05$). In both assays, significant differences were not observed between GFP cells co-cultured on the respective feeder cell lines.

Thus in this section, based on my results, I showed that this phenomenon was not specific to the particular cell-line used as the feeder layer, and could possibly be a neuronal phenomenon.

## 5.3 Summary

Taken together, in this chapter I have shown that differentiated cells were required to elicit accelerated and enhanced differentiation as undifferentiated feeders did not induce the same effect. I also showed that the underlying layer of feeder cells needed to be alive; predifferentiated feeder cells that had undergone fixation did not produce this phenomenon. Lastly, I showed that this phenomenon was not limited to the VM cell line that was used as the feeder layer, and thus could be a general phenomenon.

# Chapter 6:

## Enhanced & accelerated differentiation of NSCs is not triggered by soluble factors or by the feeder layer derived ECM

## Chapter 6:
## Enhanced & accelerated differentiation of NSCs is not triggered by soluble factors or by the feeder layer derived ECM

### 6.1 Introduction

The differentiation of neural stem cells (NSCs) is directed by intrinsic and extrinsic factors; intrinsic factors include transcription factors, proteins involved in asymmetric cell division and epigenetic modifications that have been programmed into the cell (Glaser, et al. 2007). Extrinsic factors are primarily cues provided by the cellular environment. Presumably, the NSCs seeded on top of the feeder layer in the co-culture assay have identical intrinsic properties to the cells in the control condition. As such, we hypothesize that the extrinsic environment provided by the differentiated feeder layer triggers the accelerated differentiation response. I hypothesized that accelerated and enhanced differentiation could be a result of the soluble factors released by the underlying feeder cells, the ECM deposited by the feeder cells or the direct cell-cell contact between NSCs and the feeder cells (Fig. 25). Based on this hypothesis, this chapter attempted to uncover whether the ECM or the diffusible factors released by the underlying layer of feeder cells triggered NSCs to undergo accelerated and enhanced differentiation.

The first set of experiments addressed the question whether soluble factors released into the media play a role. The roles of soluble factors or signaling molecules in neural differentiation have been extensively studied and these factors are popularly used in the induction of *in-vitro* neural differentiation (Li, et al. 2009; Lahti, et al. 2011; Altmann and Brivanlou 2001). Thus, these experiments attempted to understand if the feeder cells released signaling molecules that could elicit NSCs to undergo enhanced

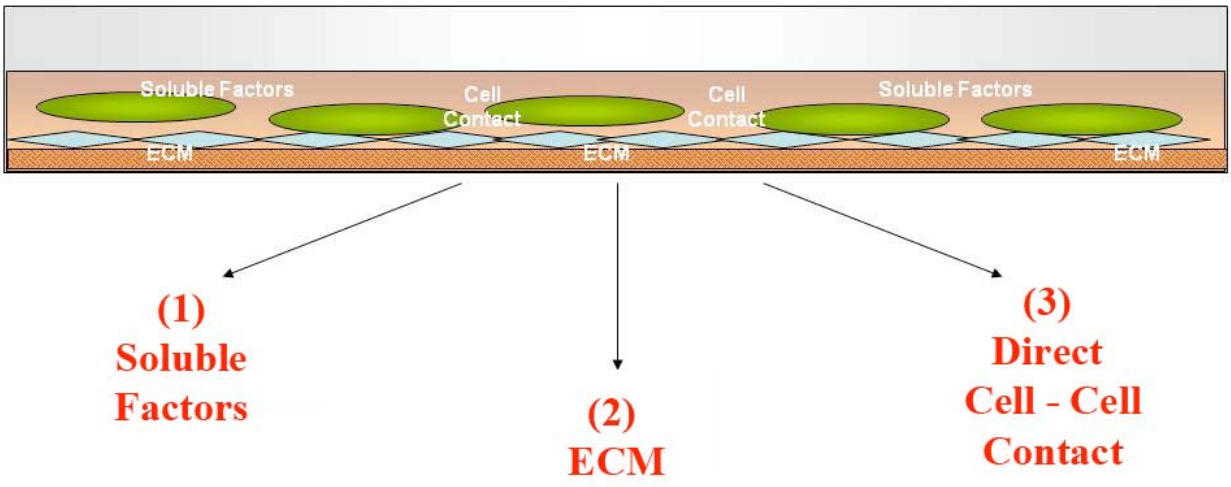


**Figure 25: Graphical representation of the hypothesis**. Accelerated and enhanced differentiation of GFP-NSCs may be a result of **(1)** Soluble factors released by pre-differentiated cells, **(2)** ECM deposited by pre-differentiated cells, or **(3)** Direct cell - cell interaction with pre-differentiated cells.

differentiation. To do this, I took the conditioned media (CM) approach and I exposed undifferentiated NSCs to conditioned medium collected from predifferentiated NSCs. The role of soluble factors was further studied by testing a concentration dependent relationship between the signaling molecules and the induced effect of enhanced differentiation.

In the second set of experiments I asked the question whether ECM deposited by feeder cells contributed to accelerated differentiation. A cell within the tissue is constantly in contact and it interacts with the ECM, which is known to have an impact on neuronal remodeling and directing differentiation (Panchision and McKay 2002; Lodish 2008). As such, could ECM molecules deposited by the underlying layer of neural cells be responsible for the induction of enhanced differentiation? To investigate this, the predifferentiated layer of cells was de-cellularized using de-cellularization methods, thus leaving the underlying ECM behind. On the assumption that some of the ECM could be disrupted in the process of de-cellularization, the ECM deposition was enhanced by the use of macromolecular crowding (Emoto 2010; Lareu, et al. 2007; Chen, et al. 2011); As opposed to the cell culture flask, the physiological environment is highly crowded, consisting of cells, ECM proteins, polysaccharides, and other types of macromolecules (Ellis 2001). The use of macromolecular crowders (MMCs) duplicates the crowded physiological space and thus limits the space available for reactant molecules. Termed as the ‘excluded volume effect’, this lack of space increases the effective concentration of reactant molecules and their thermodynamic activity, thereby resulting in increased reaction rates (Peng and Raghunath 2010; Ellis 2001). It has been shown that the deposition of ECM components is enhanced when cells are cultured with MMCs.

Utilizing this technology, I enhanced the ECM deposition of the predifferentiating feeder cells and used the generated ECM to test its ability to induce enhanced differenction after 24 hrs of culture.

The resulting data from these experiments suggested that neither soluble factors released by the feeder nor ECM deposited by the feeder layer of cells are involved in accelerated and enhanced differentiation.

## 6.2 Results

### 6.2.1 Conditioned media derived from predifferentiated NSCs does not promote accelerated differentiation

In a first experiment I tested whether conditioned media collected from predifferentiated NSCs contributed to accelerated and enhanced differentiation. The media of 7 days predifferentiated cells were collected. GFP-NSCs were seeded in 3 conditions. In the first condition (control) GFP-NSCs were seeded on laminin with fresh media; in the second condition, GFP-NSCs were seeded on laminin with conditioned media (CM); in the third condition, GFP-NSCs were seeded in the co-culture condition. GFP-NSCs were cultured for 12hrs, 24hrs and 5 days; GFP cells were assayed for neurite length and branching numbers. As seen in figure 26, after 12hrs and 24hrs of culture, GFP cells in the control condition and the CM condition have very similar morphologies and do not have extended neurite lengths or complex outgrowths with multiple branchings. In contrast, GFP cells in the co-culture condition exhibit elongated neurite outgrowths within 12 hrs of co-culture (Fig. 26C) and the effect is more pronounced after 1 day (Fig. 26F). After 5 days of culture, some GFP cells in the control condition

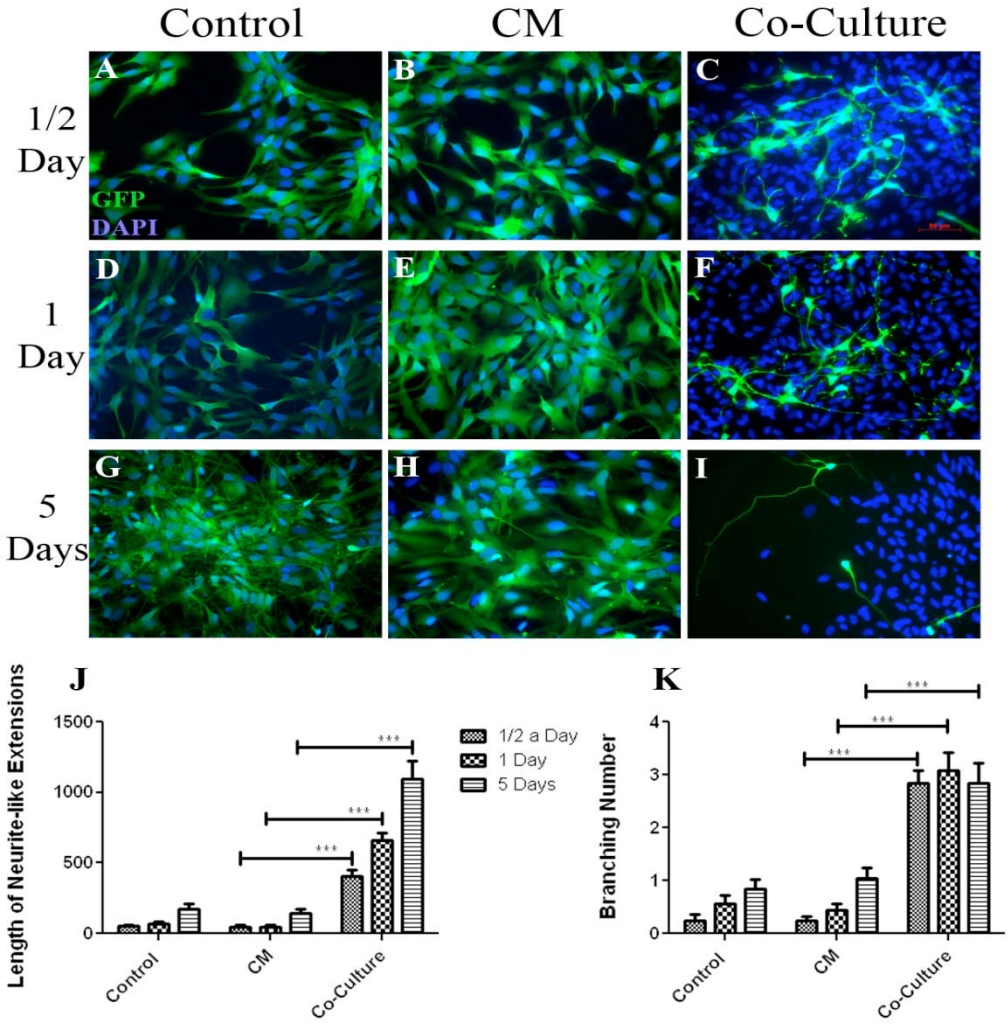


**Figure 26: Accelerated and enhanced differentiation was not induced by application of conditioned media (CM).** (**A**) GFP-NSCs cultured under control conditions for 12hrs retain NSC morphology. (**B**) In CM after 12hrs of culture, GFP-NSCs did not undergo accelerated differentiation. (**C**) Cells in the co-culture condition, however, underwent accelerated differentiation as seen in their morphology. (**D-F**) After 24hrs, neither control cells nor CM treated cells show any accelerated differentiation. Co-cultured GFP cells exhibit neuronal morphology. (**G-I**) Control cells, CM treated cells exhibit some spontaneous differentiation as observed from the extended neuritd outgrowth but they don't undergo accelerated differentiation. But co-cultured GFP cells show more enhanced differentiation as seen from the distinct neurite lengths and branchings. (**J, K**) Quantification of neurite length and number of branching points per cells. Neurite outgrowth as well as number of branching points were not increased under CM conditions. Scale bar=100. ***, $p<0.0001$.

(Fig. 26G) and the CM condition (Fig. 26H) exhibit neurite-like extensions. But the cells in 5 days of co-culture (Fig. 26I) have significantly longer neurite-like extensions with multiple neurite branchings. Quantification of the neurite length and the number of branching (Fig 26 J & K) points toward a ~10 fold increase in the neurite outgrowth in the co-culture condition as compared to the CM or the control condition ($p<0.0001$). No differences were seen between the control and the CM condition. Similarly, with regard to the branching number, there was ~10 fold increase in the co-culture condition as compared to the CM or the control condition ($p<0.0001$) just 12 hrs after culturing; no differences were observed between the CM condition and the control. Taken together, this set of data suggested that CM does not lead to accelerated and enhanced differentiation. The question then arose as to whether any possible factors that drove cells to differentiate might be too diluted in the CM. To rule out this concern, the conditioned media was collected and concentrated using an Amicon Ultra device with a 3000 Nominal Molecular Weight Limit. The CM was concentrated base on volume; 50ml of CM concentrated to 0.5ml was considered as being 100X concentration. NSCs were seeded in 20X, 10X, 5X, 1X and 0.5X concentrated CM, and undifferentiated NSCs were seeded in the respective CM concentrations. Again, cells were assayed in terms neurite-like length and number of branching point. As in the control condition, no significant neurite like outgrowth or increase in the number of branchings were observed when cells were exposed to the respective concentrations of conditioned media (Fig. 27A-F). However, significant differences were seen between the co-culture (Fig. 27G) and the control condition in terms of the length of neurite extensions ($p<0.001$) (Fig. 27H) and in terms of the branching complexity ($p<0.0001$) (Fig. 27I).

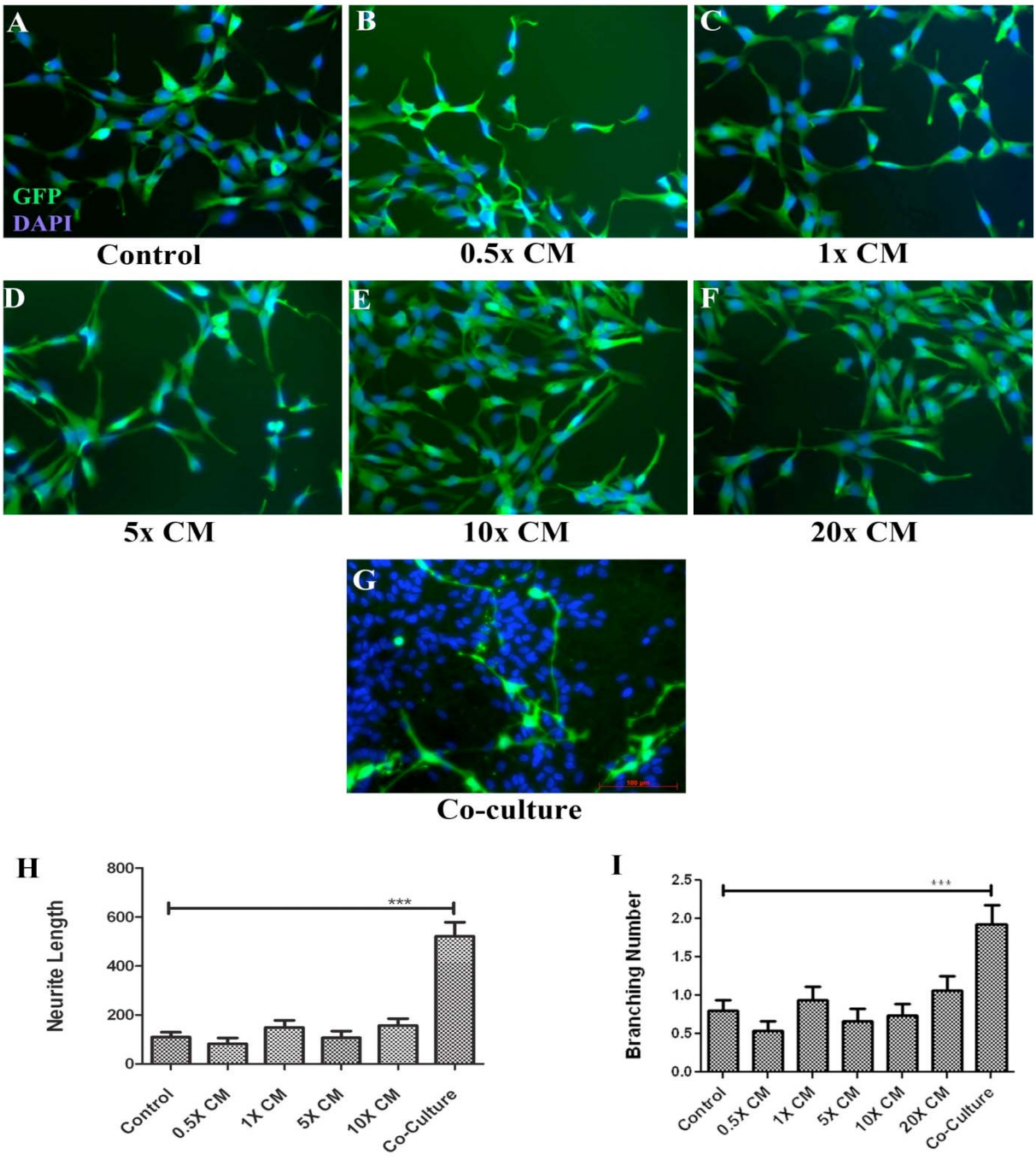


**Figure 27: Concentrated CM also does not result in accelerated differentiation.** (**A-F**) Cells in the control condition and exposed for 24hrs to CM at concentrations as indicated. No distinct morphologica differences are observed. (**G**) Co-cultured GFP-NSCs exhibit accelerated and enhanced neuronal morphology within 24hrs. **(H, I)** Quantification of neurite length **(H)** and numbers of branching points (**I**). The Neurite outgrowth as well as number of branching points were not increased under different concentrations of CM conditions. Thus, these data show that concentrating the CM does not result in comparable effects as the co-culture with predifferentiated cells. Scale bar=100. ***, $p<0.0001$.

This and the previous experiment, demonstrates that the accelerated and enhanced differentiation is unlikely a consequence of soluble factors released into the cell culture medium.

### 6.2.2 The volume of media and repeated changing of media did not influence enhanced differentiation

The following experiments act as supporting experiments to strengthen the argument that soluble factors do not trigger the accelerated and enhanced differentiation of NSCs.

Both the previous experiments completely eliminated the presence of the underlying layer of cells. If soluble factors released from the feeder cells were involved in enhanced differentiation, increasing the media volume would dilute the influence of these factors thus causing a difference in the effect of enhanced differentiation (Fig. 28 A&B). Thus, GFP-NSCs were seeded onto a layer of predifferentiated cells with media volumes of 1X (Fig. 28A) and 10x (Fig. 28B). After 24hrs of co-culture, cells were assayed for differences in neurite length and branching numbers. Cells in both condition were observed to be identical (Fig. 28 E&F) and no significant differences were observed between conditions (Fig. 28 G&H).

If the media were consistently changed, it would wash away or dilute the influence of these factors thus causing a difference in the effect of enhanced differentiation (Fig. 28C&B). Thus, to further rule-out the effect of the conditioned media, GFP-NSCs were co-cultured for 24hrs on a layer of predifferentiated cells. In the experimental condition, the media was changed every 6 hrs for 24 hrs (Fig. 28C) while in the control condition, the media remained for 24hrs (Fig. 28D). Again, the rational of the

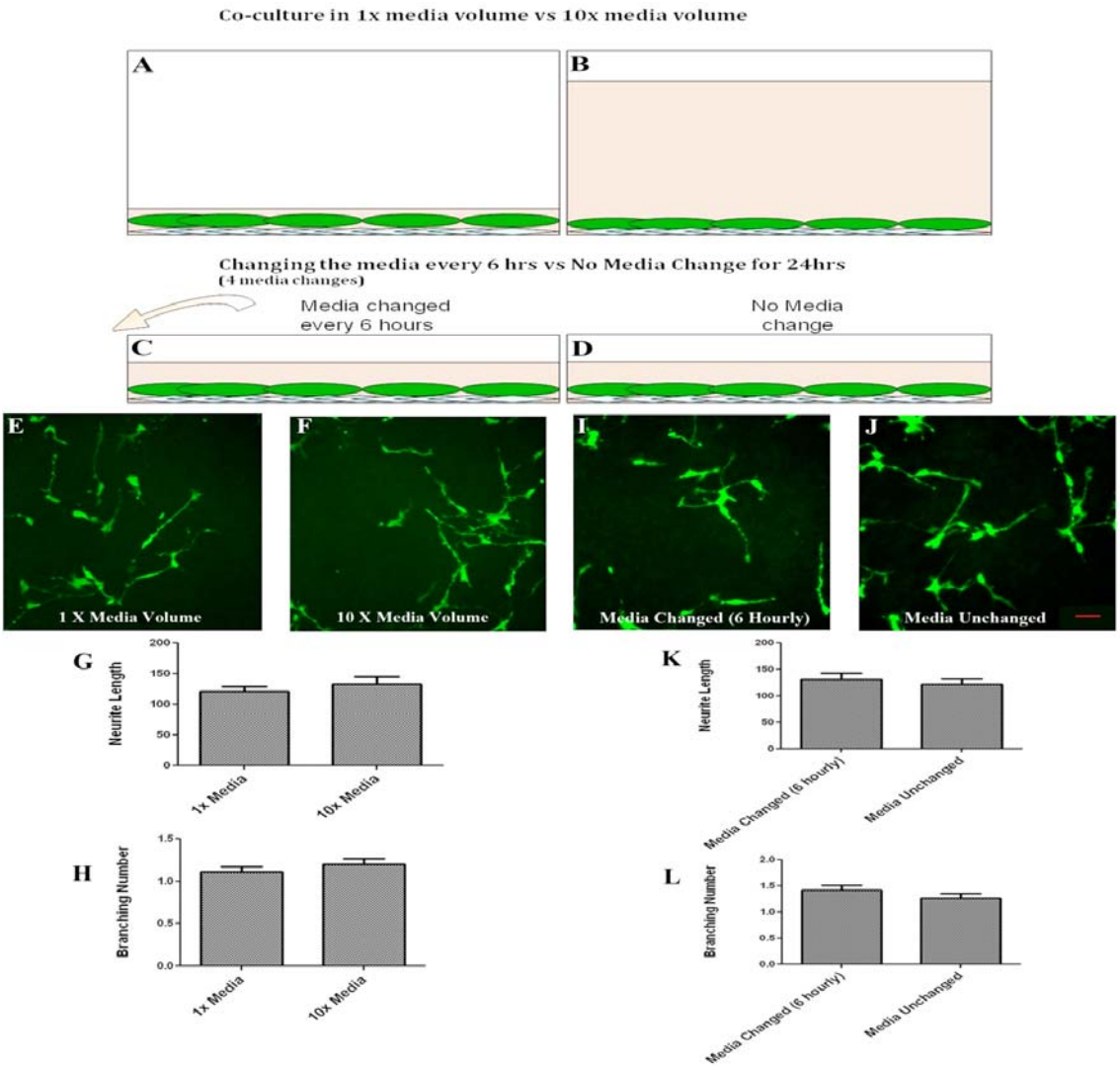


**Figure 28: Diluting possible soluble factors by increasing the media volume did not show accelerated and enhanced differentiation.** (**A-D**) Schematic representation of the experimental design. **(A, B)** GFP-NSC were co-cultured for 24hrs on a 7 day predifferentiated feeder layer under standard medium volume (**A**) and with medium 10x media volume (**B**). (**C, D**) GFP-NSCs were co-cultured for 24hrs on a 7 day predifferentiated feeder layer. The culture medium is changed every 6hrs (**C**) while the medium in (**D**) remain unchanged. **(E, F)** Co-cultured cells in both conditions (1X media and 10X media volumes) show identical morphologies with extended neurite outgrowths and complex branchings. **(G, H)** Quantification of the neurite length and the branching numbers show no differences between conditions. **(I, J)** Co-cultured cells in both conditions (media volume changed and media volume unchanged) show identical morphologies with extended neurite outgrowths and complex branchings. **(K, L)** Quantification of the neurite length and the branching numbers show no differences between conditions. Results from both experiments indicate that no changes were seen in the neurite length or the number of branches. Thus, diluting the soluble factors does not interfere with the enhanced differentiation indicating that soluble factors are probably not involved in driving enhanced differentiation. Scale bar=100.

experiment was that if soluble factors released from the feeder cells were involved then the regular changing the media would wash away or dilute the influence of these factors thus causing a difference in the effect of enhanced differentiation. After 24hrs of co-culture, no differenced were seen in in the GFP cells between both conditions (Fig. 28 I&J) and the quantification of the morphology indicated the same (Fig. 28 K&L).

### 6.2.3 ECM deposited by predifferentiated cells upon decellularization does not result in accelerated and enhanced differentiation

Having shown that soluble released by the feeder cells did not induce accelerated and enhanced differentiation, I moved on to study the role of the ECM in inducing this phenomenon. In collaboration with the Tissue Modulation Laboratory at the National University of Singapore, the 7 day predifferentiated layer of cells were decellularized using 2 established decellularization protocols : DOC and DOC DOC (Peng, et al. 2011). These 0.5% sodium deoxycholate based protocals removed the cellular layer but retains the ECM created by the cells (Peng, et al. 2011). Upon decellularization, NSCs were seeded onto the ECM left behind by the feeder layer cells and were assayed for differences in neurite like extensions, neurite branchings, the expression of NSC marker, Nestin and the neuronal makrer, β-III-Tubulin. As seen is Figure 29, the GFP-NSCs do not undergo enhanced differentiation after 24hrs of culture. Figures 29 (A-C), shows the expression of nestin in the control condition. The red staining (Fig. 29A) represents Nestin, the green staining (Fig. 29B) shows the GFP expression and the merge is shown in Figure 29C. After 24hrs of culture, all GFP cells express Nestin, as shown by the example with the white arrow. Looking at the expression of β-III-Tubulin (red)(Fig. 29D), none of the cells express the neuronal maker.

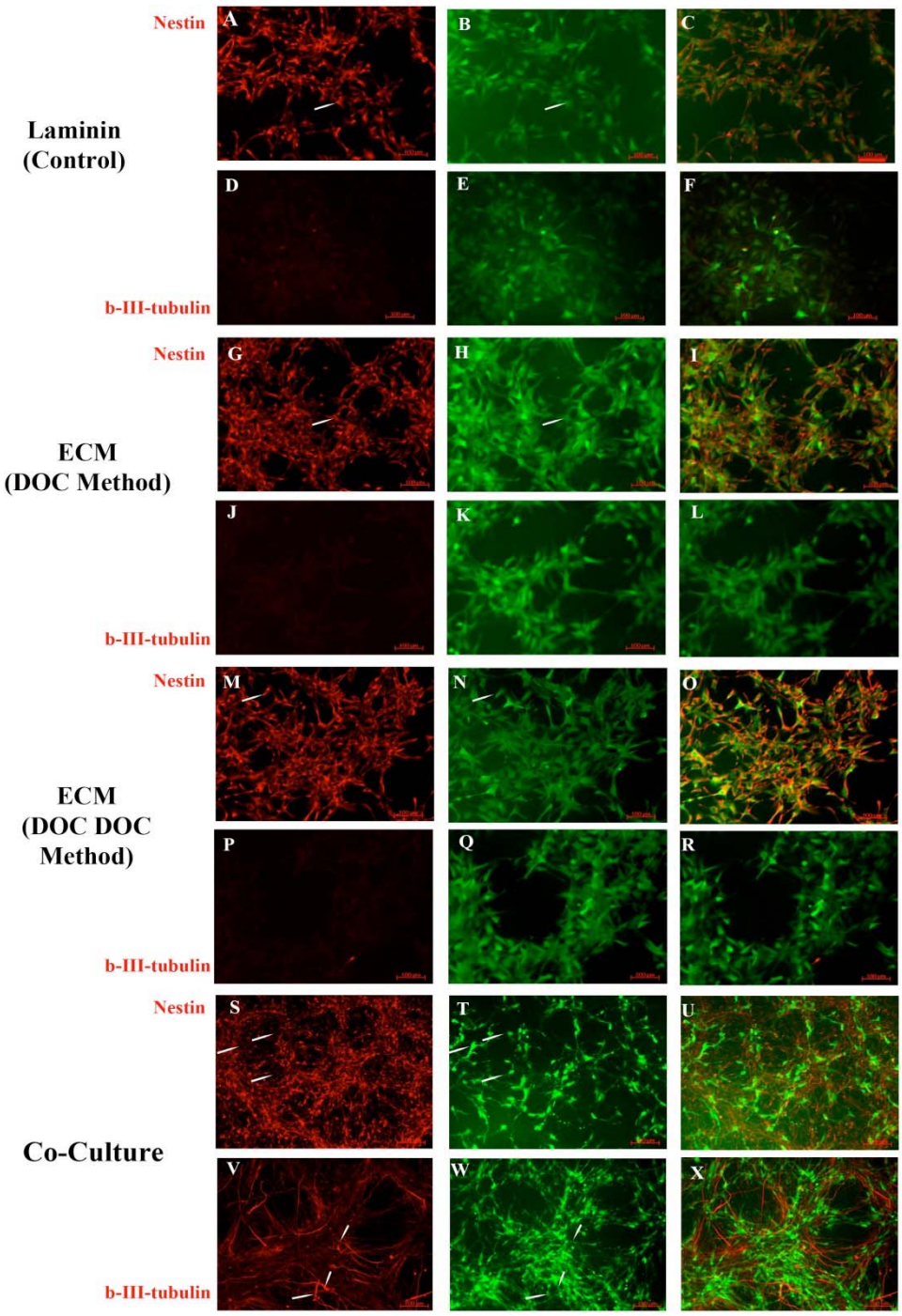


**Figure 29: ECM derived from the predifferentiated feeder cells did not induce accelerated and enhanced differentiation.** (**A-F**) GFP-NSCs seeded in the control condition for 24 hrs co-labeled Nestin and GFP (white arrow as an example) but none of the cells co-labeled with β-III-Tubulin (D-F). (**G-L**) represent cells seeded on ECM created by the DOC method (see methods) and (**M-R**) represents ECM created by the DOCDOC method. As in the control, all GFP cells seeded on ECM created by both methods (DOC & DOCDOC) have NSC like morphology, and co-expressed Nestin, but not β-III-Tubulin. (**S-X**) In the co-culture condition, GFP cells down-regulate Nestin (**S-U**) (white arrow) and up-regulate (white arrows) (**V-X**). Scale bar=50. ***, $p<0.0001$.

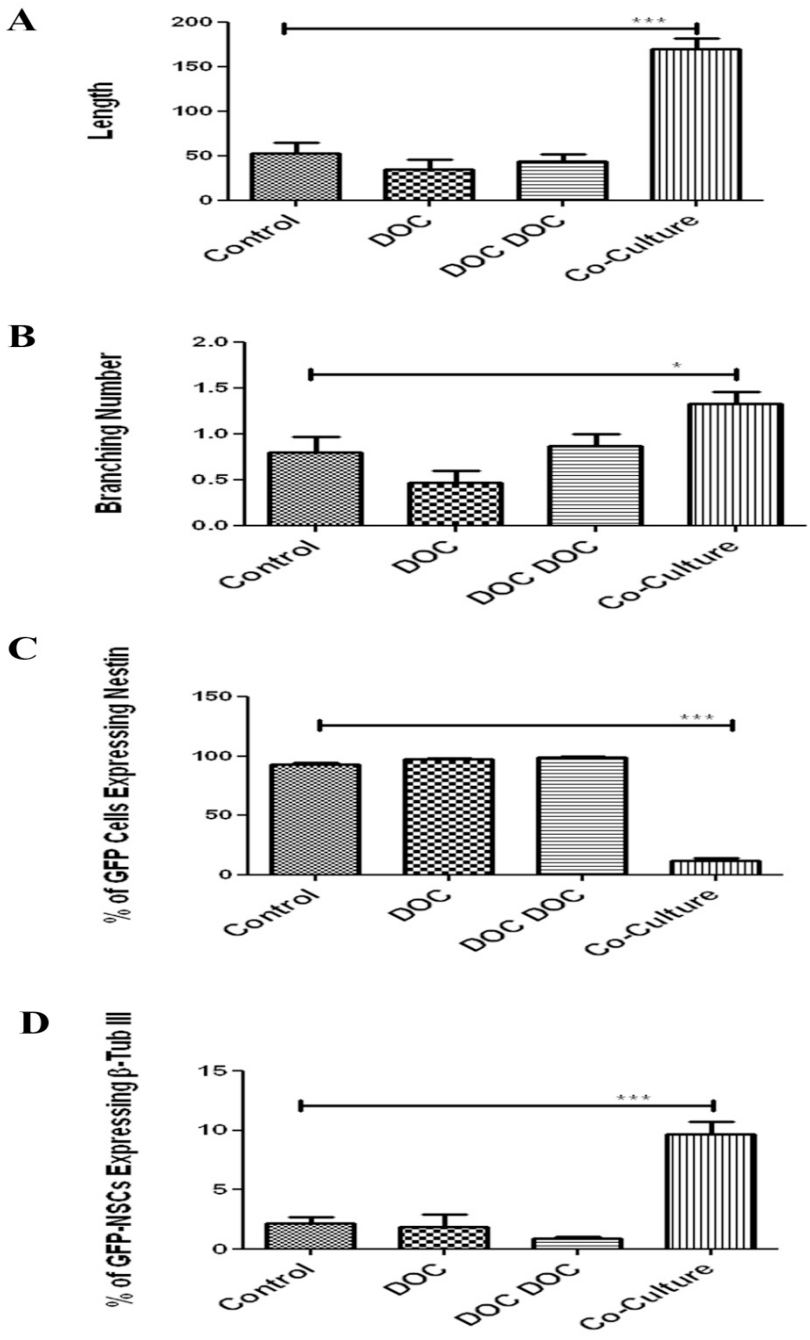


**Figure 30: Quantification of predifferentiated feeder cell derived ECM on neurite length, number of branching points, and expression of nestin and β -III-Tubulin.** (**A, B**) Quantification of morphological parameter neurite length (**A**) and number of branching points (**B**). (**C, D**) Quantification of Nestin and β -III-tubulin expression. ECM does not change the frequency of Nestin or β-III-Tubulin expressing cells and thus is probably not the factor that triggers accelerated and enhanced differentiation in co-cultured NSCs. *, $p<0.05$; ***, $p<0.0001$.

Similar to the control condition, GFP cells seeded onto DOC produced ECM did not induce accelerated and enhanced differentiation. All green cells continued to express Nestin (Fig. 29 G-I), while no cell expressed β-III-Tubulin. Similarly, GFP cells seeded onto the DOCDOC produced ECM expressed Nestin (Fig. 29 M-O) and did not express β-III-Tubulin (Fig. 29 P-R). As in the control condition, GFP cells seeded on the ECM did not exhibit neuronal morphology. In contrast, cell seeded in the co-culture condition, exhibit a neuronal morphology, down-regulated Nestin (arrows) (Fig. 29 S-U) and up-regulated β-III-Tubulin (arrows) (Fig. 29 V-X). On quantification, cells seeded on the deposited matrix closely resembled the control condition as they did not exhibit neurite outgrowth or complex neurite branchings (Fig. 30 A&B). The neurite-like extensions in the co-culture condition was more than 3 times more elongated than the control or the ECM ($p<0.0001$) (Fig. 30A). Similarly, the number of branching points was increased (1.5 fold increase; $p<0.05$) in the co-cultured cells as compared to the control condition or the cells grown on the ECM (Fig. 30B). Looking at the expression of NSC marker, Nestin, no significant difference was seen between cells in the control condition and in both the ECM conditions. However, the co-culture condition shows a~5 fold down-regulation of Nestin ($p<0.0001$) (Fig. 30C). and a marked increase in β-III Tubulin expression ($p<0.0001$)(Fig. 30D).

Thus, if the de-cellularized ECM was representative of the ECM present in the co-culture condition, then taking these results together would suggest that the ECM was not involved in the accelerated and enhanced differentiation of NSCs.

### 6.2.4 Enhanced ECM deposition through macromolecular crowding did not result in accelerated and enhanced differentiation

To further validate that the ECM was not the prime driver of enhanced differentiation, the ECM deposition was enhanced by the use of macromolecular crowders (MMCs). NSCs were predifferentiated in the presence of two different MMCs for 7 days. These were dextran sulphate (DxS) - a negatively charged macromolecule, and Ficoll - a neutral macromolecule. After 7 days of predifferentiation, the feeder cells were de-cellularized with DOC or the DOC DOC protocals. Thus, in total 6 conditions were studied:

- Control
- Ficoll Enhanced ECM - DOC Extraction
- Ficoll Enhanced ECM - DOC DOC Extraction
- DxS Enhanced ECM - DOC Extraction
- DxS Enhanced ECM - DOC DOC Extraction
- Co-culture

As seen in the uncrowded conditions, cells seeded on the deposited matrix closely resembled the control condition and did not exhibit extended neurite outgrowth. The neurite-like extensions were significantly (~ 3fold increase; $p<0.0001$;) longer in the co-culture as compared to the control condition or the ECM condition (Fig. 31A). The branching complexity was also increased as the number of branches in the co-cultured cells was significantly higher than the control or the cells grown on enhanced ECM ($p<0.05$; 1.5 fold increase) (Fig. 31B).

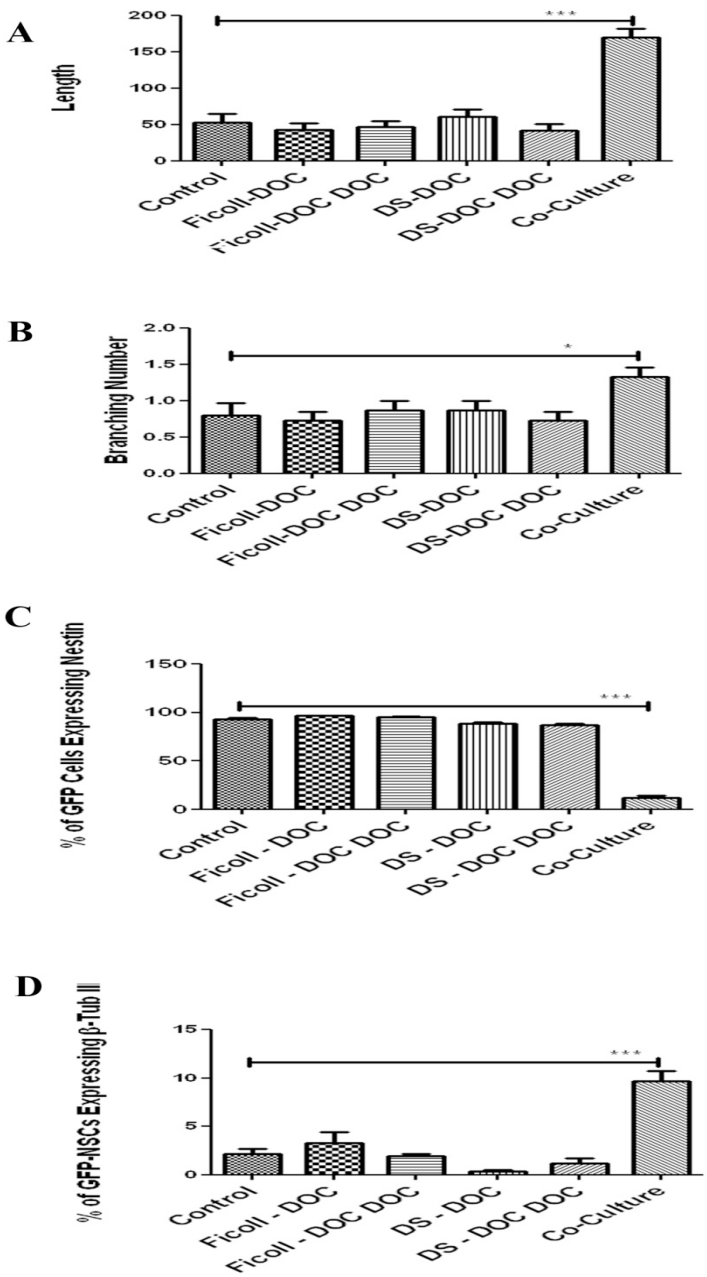


**Figure 31: ECM deposition enhanced by macromolecular crowding (MMC) does not induce accelerated and enhanced differentiation.** (**A, B**) Quantification of morphological parameters such as neurite length and number of branching points. MMC does not result in increased neurite length or number of brachng points. (**C, D**) Quantification of Nestin and β -III-tubulin expressing GFP NSCs. ECM generated under MMC conditions did not result in changes of marker gene expression on co-cultured cells. *, $p<0.05$; ***, $p<0.0001$.

Looking at the expression of NSC marker, Nestin, as in the uncrowded condition, it was observed that most of the cells retain Nestin expression and no significant difference was seen between cells in the control condition and in the ECM conditions. However, GFP cells in the co-culture condition had a ~5 fold down-regulation of Nestin ($p<0.0001$) (Fig. 31C).

With regard to neuronal marker - β-III Tubulin, as in the control condition, practically all cells seeded on the different ECMs, did not express β-III Tubulin. The co-culture condition however had more than a 2-fold increase in β-III-Tubulin protein expression ($p<0.0001$) (Fig. 31D).

Thus, based on these results, after 24hrs of culture, enhanced ECM produced through macromeolecular crowing did not result in the enhanced differentiation of NSCs.

**Summary**

This chapter attempted to understand if diffusible factors or the ECM from the underlying layer of neural cells was responsible for directing the enhanced differentiation of GFP-NSCs. The data presented here seem to suggest that the effect of enhanced differentiation is not induced by either of these extrinsic variables. Thus, direct cell-cell contact was studied.

# Chapter 7:

## Accelerated and enhanced differentiation of co-cultured NSCs is mediated by a direct cell-cell contact related mechanism

# Chapter 7:
# Accelerated and enhanced differentiation of co-cultured NSCs is mediated by a direct cell-cell contact related mechanism

## 7.1 Introduction

Knowing that extrinsic factors are involved in this phenomenon of accelerated and enhanced differentiation (Fig. 25), and having ruled-out the effect of diffusible factors released by the underlying feeder cells as well as the ECM deposited by these cells, I continued to investigate if direct cell-cell contact was a possible mechanism. Cell-cell contacts / adhesions are associated with the cytoskeleton and with cell signaling pathways (Juliano 2002), which have been shown to regulate neural development and function (de Rivero Vaccari, et al. 2007; Lee and Reichardt; Kunze, et al. 2009; Elias and Kriegstein 2008; Zhang 2010; Todd, et al. 2010; Bani-Yaghoub, et al. 1999; Elias, et al. 2007; Jaderstad, et al. 2010; Ranscht 1991; Wiertz, et al. 2011; Steinberg and McNutt 1999). I decided to use a candidate approach to identify if direct cell-cell related mechanisms were involved in accelerated and enhanced differentiation. A large number of cell-cell interactions are mediated by cell adhesion molecules (CAMs) which fall into 4 major families (Fig. 32) (Tang, et al. 2010; Lodish 2008):

1. Selectins
2. Integrins
3. Cadherins
4. Immunoglobins (Ig)

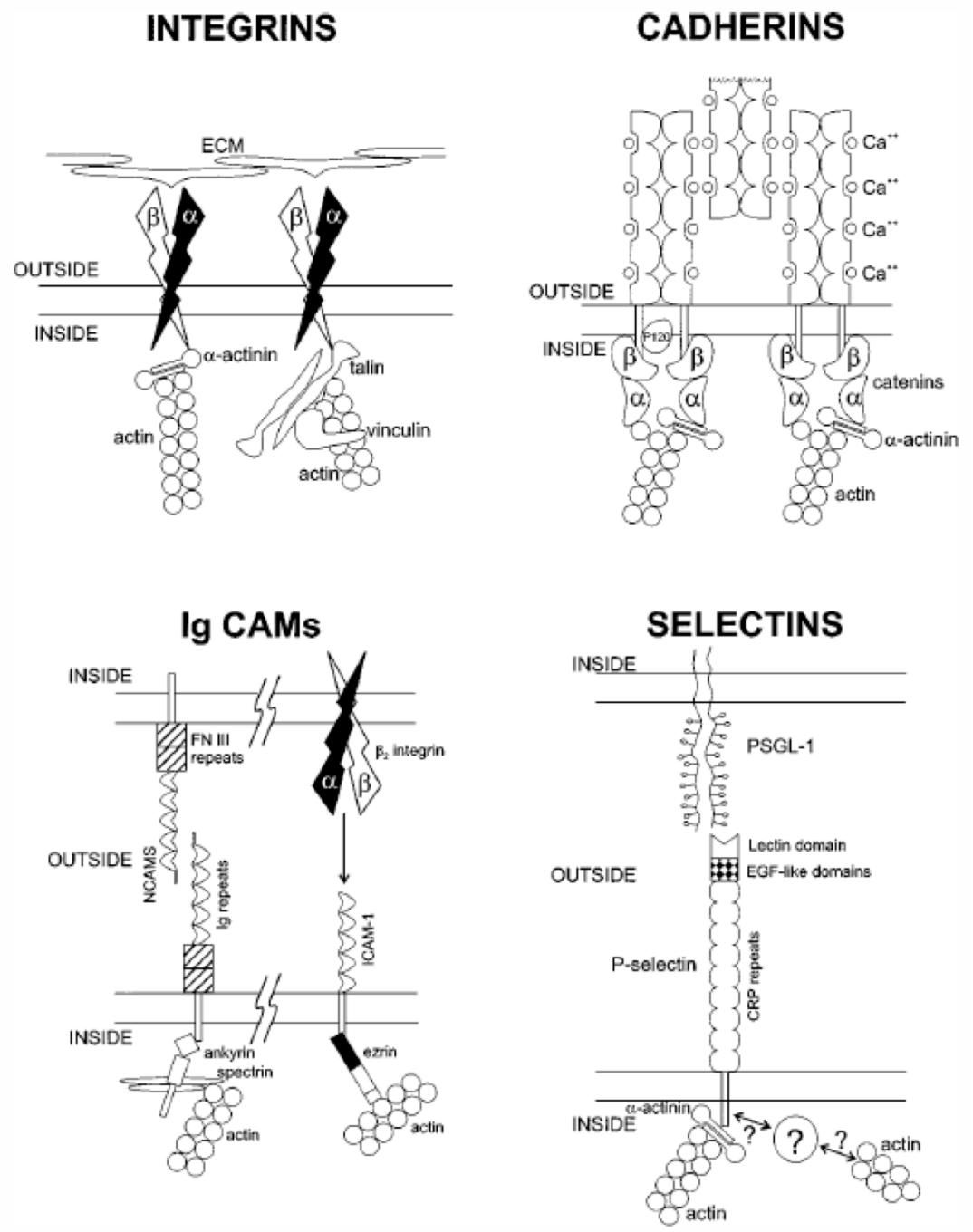


**Figure 32: Graphical representation of cell adhesion receptors and associated cytoskeletal components.** Integrins, cadherins, selectins, and Ig CAM cell adhesion receptors in association with their typical extracellular ligands and bound to the proteins that link them to the actin cytoskeleton.

These CAMs are dynamic in nature; they are broadly distributed along the plasma membrane, but upon interactions with hemophilic or heterophilic binding partners, they cluster to form discrete patches or spots called cell junctions (Mousa 2008; Lodish 2008). Cell junctions play special roles in imparting strength and rigidity to a tissue, transmitting information between the extracellular and the intracellular space. Most cell junctions consist of different CAMs and there are 3 major classes of cell junctions in the animal kingdom (Lodish 2008):

1. Tight Junctions
2. Anchoring Junctions
3. Gap Junctions

Tight junctions are generally not found in the neural cells (Lodish 2008). Gap junctions and anchoring junctions, on the other hand, are extensively found in the brain (Kunze, et al. 2009; Elias and Kriegstein 2008; Dermietzel and Spray 1993; Zhang 2010; Nakase 2004). Anchoring junctions in cell-cell interactions are mediated by Cadherin molecules and the most rampant cadherins in the brain are N-Cadherins (Ranscht 1991). Thus, in attempting to identify the mechanism of action, we chose to pharmacologically inhibit gap junctions and N-Cadherins as the first choice of candidates.

My results indicated that the inhibition of both, gap junctions and N-Cadherins inhibited the accelerated differentiation of co-cultured NSCs.

## 7.2 Results

### 7.2.1 Inhibition of Gap Junctions may inhibit accelerated and enhanced differentiation

The rationale of this experiment was that if gap junction formations between the feeder layer and the co-cultured GFP-NSCs were responsible for accelerated and enhanced differentiation, inhibiting them would inhibit the accelerated and enhanced differentiation of NSCs. Thus, gap junctions were inhibited pharmacologically by the combination of two gap junction antagonists: carbenoxolone (CBX), and 18β-glycyrrhetinic acid (18-GA) (Lodish 2008; de Rivero Vaccari, et al. 2007). On performing a kill curve (data not shown) to estimate the maximum drug concentration that induced cell death, a cocktail consisting 60uM of CBX and 75uM of 18βGA was used. GFP-NSCs were co-cultured onto a layer of predifferentiated cells with or without the presence of the blockers. GFP-NSCs were also seeded in the control condition with or without the presence of the blockers. After 24hrs of culture, cells were first assayed for neurite-like extensions and the number of branchings per cell. Without the presence of gap junction blockers, in comparison with the control condition (Fig. 33B), as expected co-cultured NSCs expressed neuronal morphology with extended neurite out-growth (Fig. 33A). In the presence of the gap junction blockers, co-cultured cells exhibit a truncated morphology with a 6-fold reduction of neurite length ($p<0.0001$) (Fig. 33 C, E) and a 2-fold reduction in the number of neurite branchings ($p<0.0001$) (Fig. 33F) The expression of the NSC marker Nestin was up-regulated by 4-folds as compared to the cells cultured without blockers ($p<0.0001$) (Fig. 33G). However, looking at the control condition, GFP-

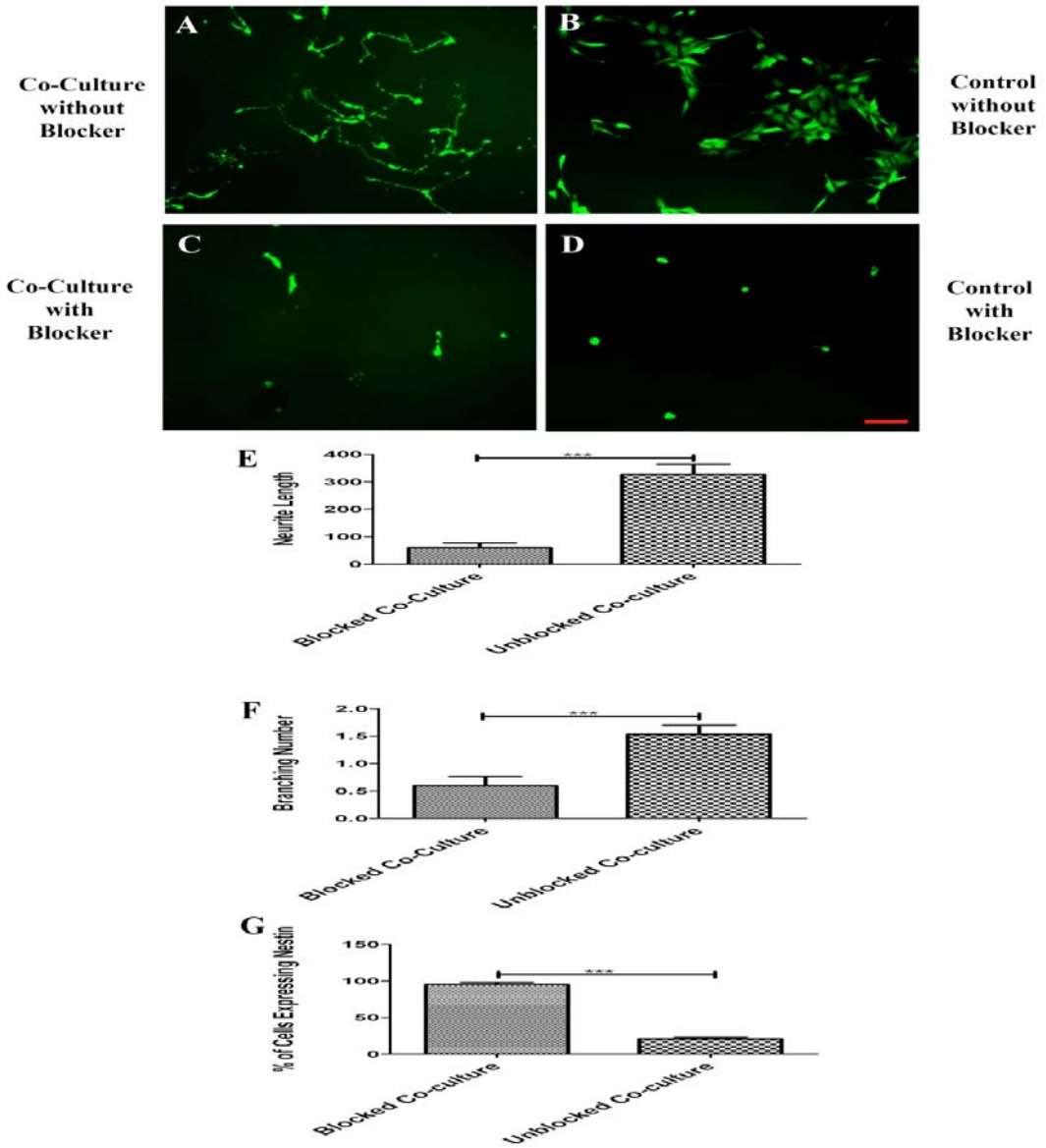


**Figure 33: Pharmacological inhibition of gap junctions inhibits accelerated and enhanced differentiation.** (**A, B**) The unblocked co-culture condition and the unblocked control condition. GFP-NSC in the co-culture condition exhibit the neuronal morphology while the control condition continued to retain NSC morphology. **(C)** In the co-culture condition with the presence of the blocker, the accelerated differentiation of NSCs is inhibited. Though the number of cells attached to the feeder is reduced, the cells that are attached do not show significant neurites extentions as compared to the unblocked condition. **(D)** In the control condition with the blocker, the cell numbers is also reduced and the morphology of the cell do not resemble the control. **(E - G)** Quantification of morphology shows significant differences in terms of neurite length, branching complexity and Nestin expression of the co-cultured cells with and without the blocker. Scale bar=100μm. ***, $p<0.0001$.

NSCs incubated with blockers had a morphology similar to that of the inhibited cells in the co-culture condition (Fig. 33D). Furthermore, the cell numbers in the control condition were significantly less than the control condition without the blockers.

These results indicate that the inhibition of gap junctions also inhibited accelerated and enhanced differentiation. However, the lack of cell numbers and the retardation of cell morphology in the control condition with inhibitors seem to indicate that the effect could also be induced due to the toxicity of the inhibitors. Alternatively, the gap junction inhibitors could be inhibiting a more fundamental cellular process, which disrupts survival and enhanced differentiation as a secondary effect. Therefore, more experiments need to be conducted so as to elucidate the role of gap junctions.

### 7.2.3 Inhibition of N-Cadherin inhibits accelerated and enhanced differentiation in a dose dependent manner

As in the previous experiment, the rationale of this experiment was that if N-Cadherin (N-CAD) interactions between the feeder layer and the co-cultured GFP-NSCs were responsible for accelerated and enhanced differentiation, inhibiting N-CAD would inhibit the accelerated and enhanced differentiation of NSCs. Thus, N-CAD was inhibited by using an anti N-Cadherin (human specific) antibody to block its function (Wiertz, et al. 2011; Meyer, et al. 1992). GFP-NSCs were co-cultured onto a layer of predifferentiated NSCs with and without the presence of the respective blocking antibody. In a dose dependent manner, the following blocker dilutions of antibody was used: 1:20 (Fig. 34 C & G), 1:100 (Fig. 34 D & H), 1:200 (Fig. 34 E & I), 1:800 (Fig. 34 F & J). In the unblocked condition, within 24hrs of co-culture GFP cells underwent

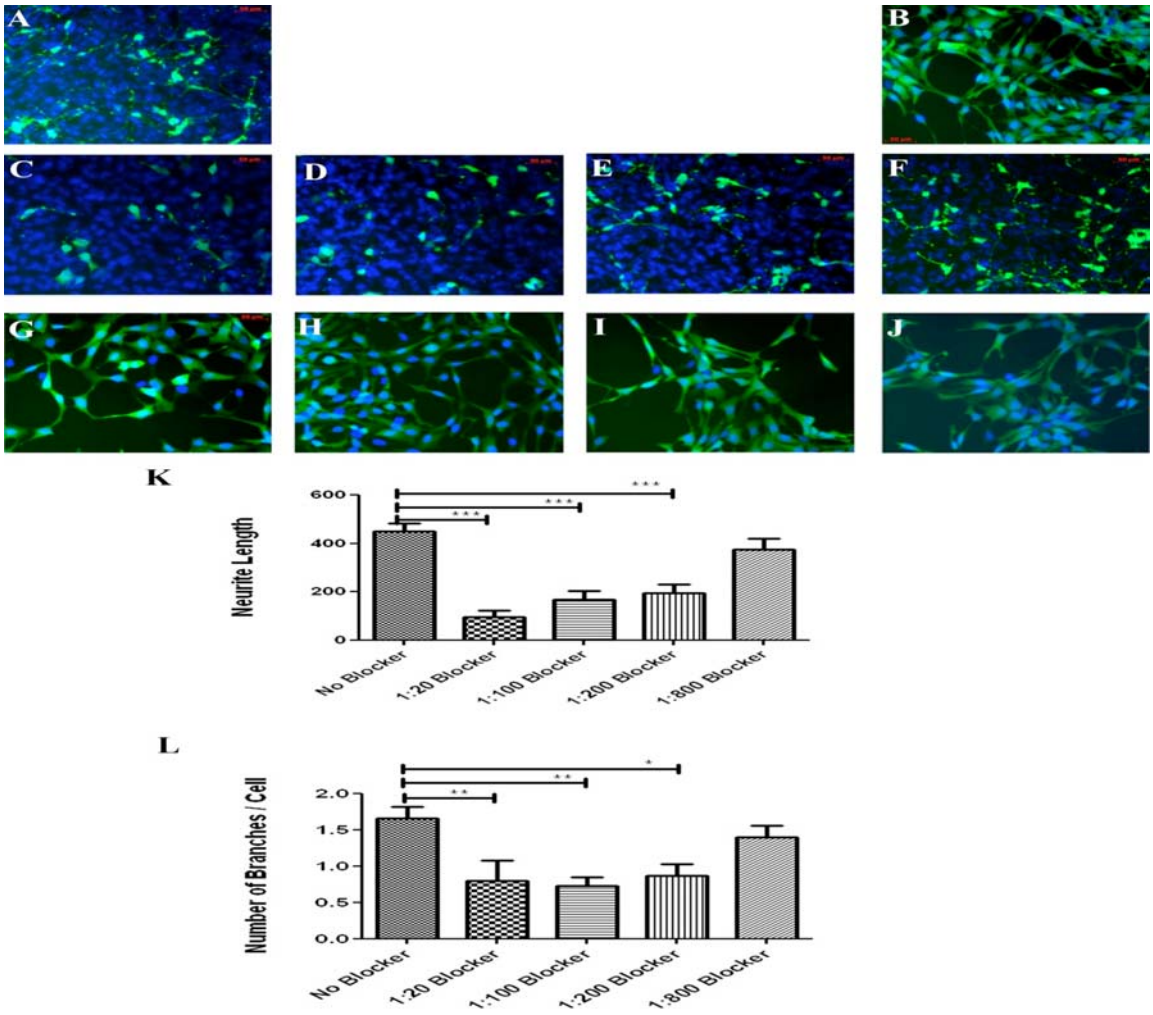


**Figure 34: N-Cadherin blocking antibodies inhibit accelerated and enhanced differentiation. (A)** Co-culture condition without blocking. GFP-NSCs exhibit enhanced differentiation after 24hrs of co-culture (**B**) Control condition without co-culture and blocking exhibit NSC morphology. (**C-F**) Cells grown under co-culture conditions incubated with 1:20 (**C**), 1:100 (**D**), 1:200 (**E**) and 1:800 (**F**) of the blocking antibody are shown. With the increase in blockers concentrations, there was a reduction in accelerated and enhanced neuronal differentiation as seen by the change in morphology. Enhanced differentiation was completely inhibited in the 1:20 dilution while enhanced differentiation was not inhibitered for most cells with 1:800 dilution of the blocking antibody. (**G-J**) GFP-NSCs grown in the non co-culture 'control' conditions with 1:20 (**G**), 1:100 (**H**), 1:200 (**I**) and 1:800 (**J**) of the blockers. No major morphological differences were noticed. (**K, L**) Quantification of neurite length number of branche are shown. At 1:20 dilution of blocking antibody, a 3-fold reduction in neurite length was observed. With increasing dilutions, the neurite length increased and no significant difference was observed in the 1:800 blocker. Similarly, significant differences in the number of branchings were noticed in the GFP cells when co-cultured in the presence of blockers but no significant difference was observed in the 1:800 blocker. These data show a dose dependent reduction of neurite outgrowth and numbers of branches per cell. Scale bar=100μm. *, $p<0.05$; **, $p<0.01$; ***, $p<0.0001$.

accelerated differentiation, as noticed from the cell morphology (Fig. 34A); cells in the control condition continued to exhibit NSC morphology after the same duration of culture (Fig. 34B).

In the blocked co-culture condition (Fig. 34 C-F), the differentiation of GFP cells seemed to be inhibited. As the dilution of the antibody increased (or concentration decreased), more cells exhibited a differentiated morphology in terms of neurite length and the number of branching per cell. As seen from Figure 34C, none of the co-cultured GFP cells exhibited neuronal morphology. As the dilution increased, more cells exhibited neuron like morphologies (Fig. 34 E & F). In terms of the elongation of the neurite-like extensions, a significant difference was observed between the non-blocked co-cultured GFP cells and the cells co-cultured in the presence of 1:20, 1:100, and 1:200 dilutions ($p<0.0001$) (Fig. 34K), thus indicating a dose dependent effect of the blocking antibody on accelerated differentiation. Similarly, when the number of branchings were quantified, a ~ 2 fold difference was seen between the co-cultured NSC with and without the inhibitors (Fig. 34L). GFP-NSCs cultured in the control condition (Fig. 34G-J), with the presence of different concentrations of the blockers continued to exhibit NSC like morphologies after 24hrs of culture, as in the control condition (Fig. 34B).

These results clearly indicate that the blocking of N-CADs also inhibited accelerated and enhanced differentiation of co-cultured NSCs. The inhibited cells continued to exhibit NSC like morphology and no increase in cell death was observed.

## 7.3 Summary

This chapter attempted to point towards the mechanism governing the accelerated and enhanced differentiation of co-cultured GFP-NSCs. Taking the data presented suggests that both the inhibition of gap junctions and N-CAD s prevented the accelerated and differentiation suggesting that gap junctions and N-Cadherin are important mediators in this process. However, their mechanisms of action could be different.

# Chapter 8:

# Generation and validation of a DDC-GFP Promoter Reporter NSC cell-line

## Chapter 8:
## Generation and validation of a DDC-GFP Promoter Reporter NSC cell-line

### 8.1 Introduction

Having developed and characterized a co-culture system that enables the accelerated and enhanced differentiation of NSCs, this technology was applied as a tool to validate a promoter-reporter cell line.

To study dopaminergic (DA) and noradrenergic (norepinephrinergic) differentiation of NSCs possibly in a high content screening manner, without the need of extraneous immunocytochemistry, I designed and generated a promoter reporter cell-line that would express the in-vivo marker GFP in cells activating the DOPA decarboxylase (DDC) promoter. The ultimate goal would be to use this line to perform efficient testing of different compounds and pharmacological moieties that would be involved in regulating DDC. DDC is an enzyme that is involved in converting DOPA into dopamine (Siegel and Agranoff 1999). In conjunction with tyrosine hydroxylase (TH), DDC is used as a marker for DA neurons as well as noradrenergic neurons (Chatelin 2001; Siegel and Agranoff 1999) (Fig. 35).

Figure 2 depicts the strategy. A part of the non-neuronal exon 1 and neuronal exon 1 (promoter region of the DDC gene) was conjoined with exon 2. Then, via the Gateway recombineering system, this entire construct together with an eGFP containing vector was cloned into a single vector (destination vector). This vector was infected into NSCs.

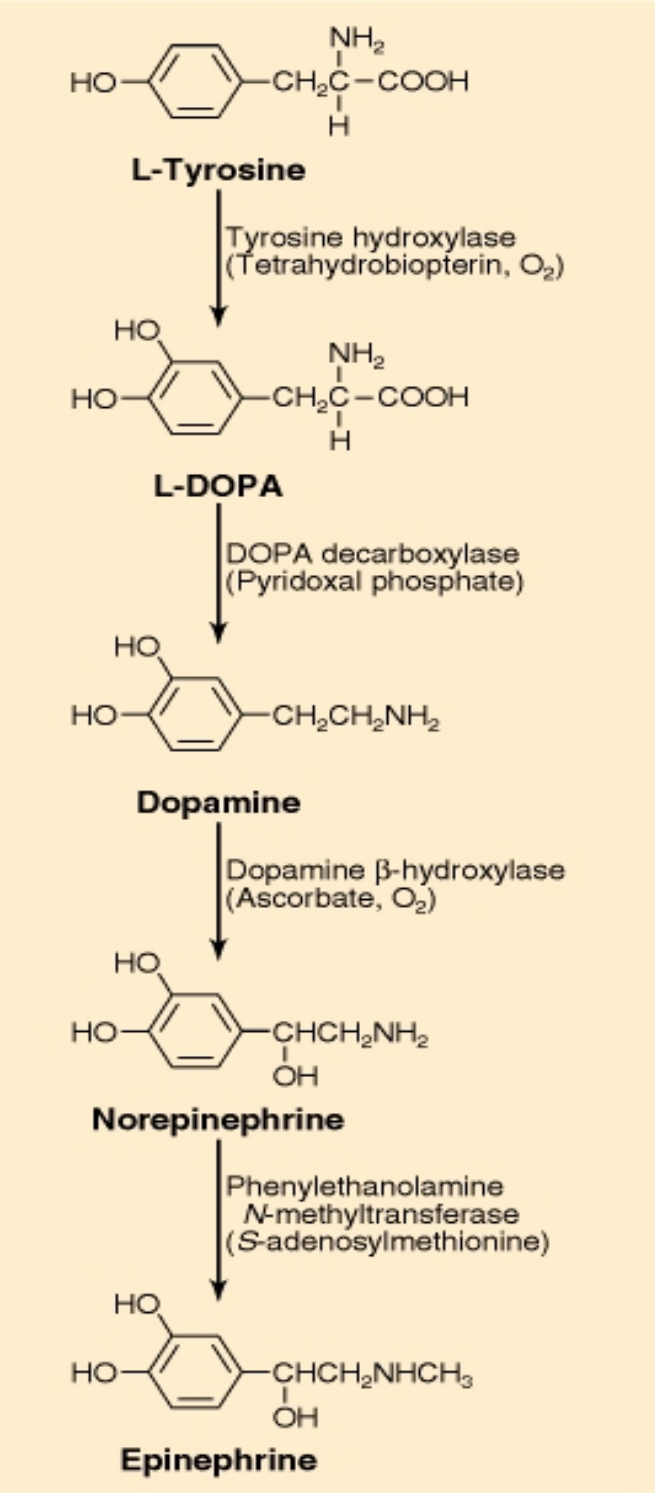


**Figure 35: Biosynthetic pathway for catecholamines such dopamine, norepinephrime and epinephrine synthesis**. DDC is necessary for the synthesis of DOPA to dopamine.(Siegel and Agranoff 1999)

Having infected and selected NSCs for the respective vector, the task at hand was to validate that the expression of GFP coincides with the expression of DDC. As this system does not permit the post-translational regulation of DDC, which could be of importance in DDC protein expression, our cell line had to be validated (Kang and Han 2011; Lodish 2008).

## 8.2 Results

### 8.2.1 Utilization of novel co-culture system for the validation of DDC-GFP NSCs

DDC-GFP NSCs were differentiated via the standard cell culture method and a 1.5 fold increase (not shown) in GFP expressing cells was seen after 7 days (Fig. 36 A, B). However, it was noted that a small population of NSCs expressed a basal level of GFP even in the undifferentiated state (Fig. 36A). DDC being a neuronal phenotypic marker generally should not be expressed in the undifferentiated state. This population of GFP expressing cells were sorted out of the cell population, thus giving us a total of 3 cell populations:

1. NSCs expressing basal levels of GFP (GFP '+'ve)
2. NSCs not expressing basal levels of GFP (GFP '-'ve)
3. Mix population of NSCs (Unsorted)

The presence of these 3 cell populations led to the following two questions that needed to be tested. Firstly, could the cells that possed a basal endogenous expression of DDC be progenitors to catecholamines neurons or are they false positive cells? Secondly, which

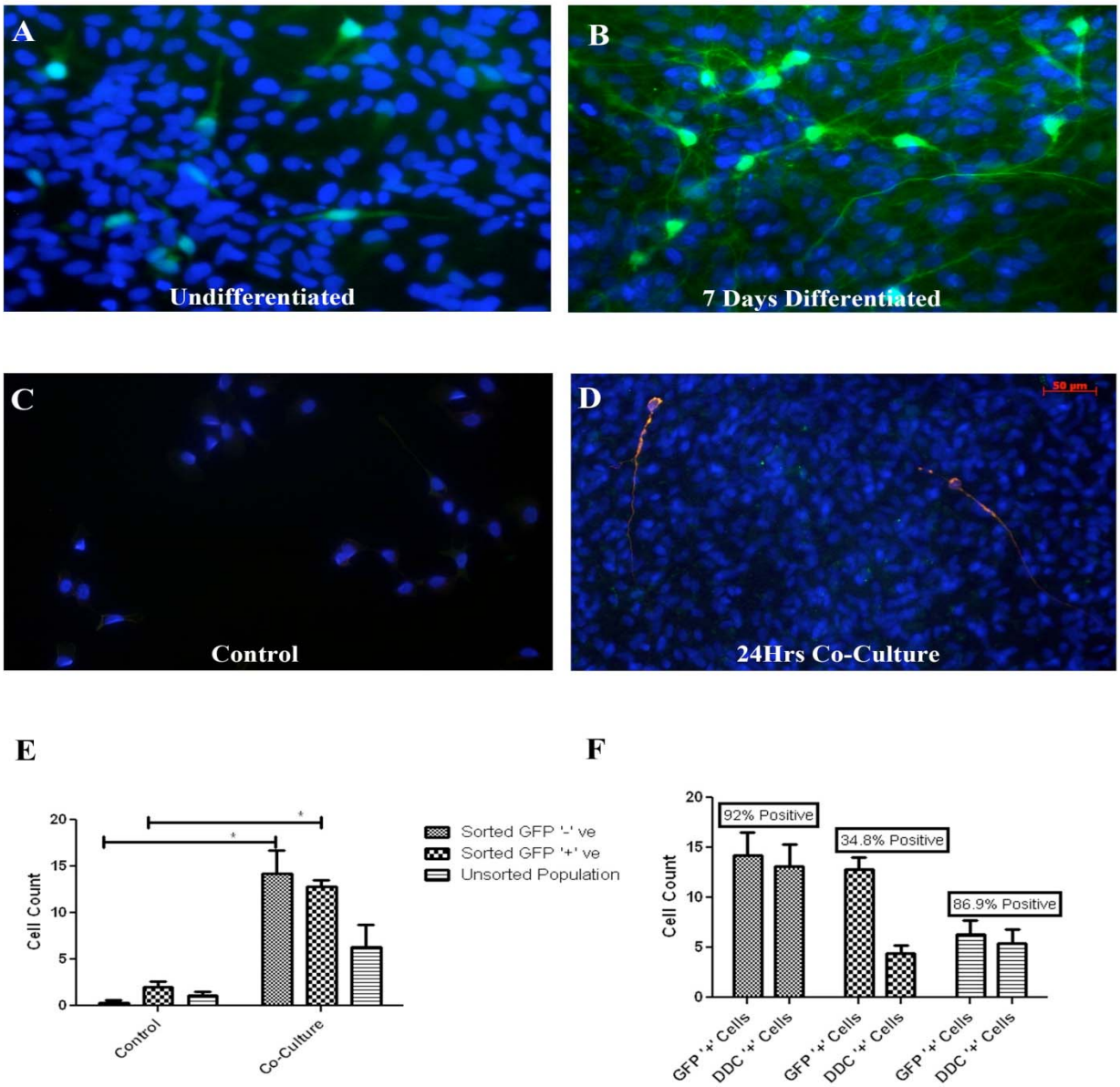


**Figure 36: Application of novel NSC co-culture system: Validation of DDC-GFP line.** Co-culture system used as a tool for the rapid validation of DDC-GFP NSCs. **(A, B)** DDC-GFP NSCs were differentiated under non co-culture conditions. (A) Undifferentiated NSCs and (B) DDC-GFP NSCs differentiated for 7 days. Cells were observed to have a basal expression of GFP in the undifferentiated state. GFP cells were sorted out of the total population of cells so as to give a population of GFP + cells and a population of GFP – cells. Each population of cells was co-cultured for 24hrs on a layer of predifferentiated cells. **(C, D)** Using my novel co-culture system, DDC-GFP NSCs expressed GFP and co-labeled for α DDC within 24hrs. **(E)** The up-regulation of GFP on the co-cultured GFP-NSCs was significantly higher than the control. **(F)** To understand which of the cell populations (GFP+, GFP-, or mixed) was best as a promoter-reporter cell line, the co-culture system identified GFP- as the most optimal cell-line that had the highest co-labeling of GFP-DDC (within 24hrs of culture). Scale bar=50µm. *, $p<0.05$

of these populations would best represent a DDC-GFP NSC line in terms of GFP-DDC co-labeling?

Using our novel co-culture method, I attempted to answer the above questions. Each of the cell populations were co-cultured with a 7 day predifferentiated layer of cells for 24hrs; in the control condition, cells were cultured on laminin for the same duration of time. Cells were assayed for the expression of GFP as well as the native DDC protein via immunocytochemistry.

Looking at the GFP '-' population of cells, after 24 hrs of culture none of the cells in the control condition expressed GFP or labeled α-DDC (Fig. 36 C & E). In contrast, cells in the co-culture condition expressed GFP, which co-labeled with α-DDC staining (red) (Fig. 36 D). Approximately, 15% of the total GFP '-' cell population expressed GFP after 24hrs of co-culture (Fig. 36E) and 92% of these cells co-labeled with α-DDC (Fig. 36 F). In the GFP '+' population of cells, though ~12% of cells expressed GFP after 24hrs of co-culture (Fig. 36E), only 34.8% of these cells co-labeled with α-DDC, thus suggesting that the presence of false positive cells. In the unsorted population, there was no significant increase in GFP after 24hrs of co-culture.

Taken together, the results seem to suggest that the basal level of GFP on NSCs represented a population of false positive cells; the negatively sorted GFP-NSCs most faithfully reported DDC expression.

## 8.3 Summary

This chapter set out to put this co-culture NSC differentiation system to an application. A DDC-GPF promoter-reporter cell-line was designed and created; this cell-line was effectively validated for its expression of DDC via this co-culture system. Based

on this experiment, the population of NSCs sorted for GFP negativity (GFP '-' ve) were most promising. It is worth to note that in general, it would take ~7-14 days (de Rivero Vaccari, et al. 2007) with a standard cell culture approach to validate a cell line such as this, but the co-culture system with the accelerated differentiation allowed me to perform this validation within 24hrs of differentiation.

# Chapter 9:

# Discussion

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## Discussion

The premise of this study was that the present standard cell-culture method is sub-optimal and thus inefficient and ineffective in the differentiation of neural stem cells (NSCs) (Teo 2010; Blow 2009) to neurons. I hypothesized that the reason for this was because cells did not receive the necessary extrinsic cues as they do in the *in-vivo* system of the brain (Meyer, et al. 2009). If these cues were provided in a favorable manner, neuronal differentiation could be made more efficient and effective. I thus generated a novel cell culture system using a feeder layer that consisted of predifferentiated NSCs. The feeder layer consists of cells that spontaneously differentiated to various lineages and were at different stages of development; these cells naturally released soluble factors (Tang, et al. 2010; Zhu, et al. 2008), deposited their own ECM (Lareu, et al. 2007; Silva, et al. 2009), exerted mechanical stimuli to each other and formed their own unique nanotopography (Teo 2010; Avvisato, et al. 2007). As such, the feeder layer replicates some of the extrinsic cues present in the *in-vivo* system. Based on the exhibition of neuronal morphology, the down regulation of NSC markers and the up regulation of neuronal markers, our hypothesis was validated as NSCs underwent accelerated and enhanced neuronal differentiation. I showed that the feeder layer had to be predifferentiated and had to be alive so as to induce accelerated and enhanced neuronal differentiation. The effect was not caused by the soluble factors released or the ECM deposited by the feeder layer. Direct cell-cell contact was necessary to mediate accelerated and enhanced neuronal differentiation.

**The standard conditions for NSC culture**

I have shown that the standard cell-culture method, i.e. differentiation through removal of mitogenic factors is sub-optimal in achieving efficient and effective Neural Stem Cell (NSC) differentiation. NSCs cultured by the *in-vitro* standard cell culture method were characterized by the expression of NSC markers, the expression of neuronal markers and the potential to elicit action potentials. Most NSCs expressed the respective NSC markers such as SOX 2, Nestin, Musashi and GFAP, and were actively cycling as they expressed Ki 67. Upon the removal of the mitogenic stimulus through growth factors, cells started to differentiate, and most cells exited the cell cycle within 4-5 days of differentiation. These cells also up-regulated genetic markers for the 3 neural lineages: neurons, astrocytes and oligodendrocytes, indicating a tripotential neural differentiation capability which is characteristic of NSCs (Glaser, et al. 2007; Demir, et al. 2009). However, in the standard culture conditions most cells tended to retain NSC marker expression. In addition, only a relatively small percentage (about 15%) of cells expressed neuronal markers after differentiation. Furthermore, when differentiated cells were assayed through $Ca^{2+}$ imaging, the data indicated that although cells showed an increase in $Ca^{2+}$ intensity upon the changing of the membrane potential, the measured amplitude seemed to indicate that the cells were not fully mature and functional neurons. Neuronal cells usually have an influx of $Ca^{2+}$, with a $\Delta F/F$ value of about 4 to more than 10 (Goldman 1998; Bito, et al. 1997; Takahashi, et al. 1999). But even after 2 weeks of differentiation, cells grown and differentiated *in-vitro* had a maximum intensity of 1.8, thus indicating immaturity.

To further elucidate the electrical maturity of differentiated cells, Patch Clamping analysis was performed. The objective of this experiment was to understand if the cells can generate action potentials. Results indicated that as in the undifferentiated state, even after 14 days of differentiation, action potentials could not be observed. When probed with in a voltage-clamp configuration, results indicated a lack of inward current. The inward current is generally due to the inward flux of $Na^+$ ions, which is also responsible for the generation of any action potential. Thus taken together, the data may suggest that these cells were either not capable of regulating the necessary $Na^+$ channels or that the conditions were not conducive for the expression of the respective $Na^+$ channels after 14 days of differentiation and thus cells were not able to generate action potential (Bean 2007; Jelitai, et al. 2007; Bear, et al. 2007). However, $Na^+$ currents have been reported to appear early in neuronal differentiation (Jelitai, et al. 2007); thus, the lack of action potentials substantiates the fact that our differentiating cells were not functionally mature.

**The extrinsic cues involved in neuronal differentiation**

During development and adult neurogenesis, NSCs migrate and interact with other neural cells of different lineages and stages of development (Schumm, et al. 2003; Ditlevsen and Kolkova 2008; Ditlevsen, et al. 2008; Elias, et al. 2007; Tsai and McKay 2000; Chao, et al. 2009), and the micro-environment surrounding these cells (Silva, et al. 2009; Blow 2009; Meyer, et al. 2009; Even-Ram, et al. 2006; McNamara, et al. 2010), before acquiring their final differentiated fate. Such cues are probably insufficiently represented in the *in-vitro* differentiation system. Apart from direct cell-cell interactions, the natural micro-environment consists of ECM, diffusible / soluble factors, and the mechanical / nanotopographical architecture with which cells interact (Teo 2010;

Avvisato, et al. 2007; Alenghat and Ingber 2002). If these factors could be included in an *in-vitro* culture system, in a favorable manner, NSCs would probably more efficiently and effectively differentiate into mature neurons (Teo 2010).

**Replicating the *in-vivo* environment through a co-culture system resulted in accelerated and enhanced differentiation**

In order to provide such extrinsic cues, a co-culture system using predifferentiated NSCs was set-up and utilized for further studies. NSCs tagged with GFP were co-cultured on a feeder layer of untagged predifferentiated NSCs and cultured between 12hrs and 7 days in differentiation conditions. Knowing that spontaneously differentiating NSCs differentiate to a heterogeneous population of neural cells (Glaser, et al. 2007; Reynolds and Weiss 1992), a predifferentiated NSC feeder layer was utilized as it could possibly provide dynamic interactions comprising of direct cell-cell interaction, cell-ECM interactions and interaction with different soluble factors. They would naturally also exert mechanical stimuli to each other and form their own unique nanotopography (Teo 2010; Avvisato, et al. 2007). As such, the feeder layer could replicate some of the extrinsic cues present in the *in-vivo* system in a culture dish.

I hypothesized that if cells received the necessary extrinsic cues as they did in the *in-vivo* system, neuronal differentiation could be made more efficient and effective. This hypothesis was validated because the co-cultured GFP-NSCs showed accelerated and enhanced differentiation. When compared to the control condition (standard cell culture system) co-cultured NSCs rapidly (within 12hrs) exhibited a neuronal morphology, down regulated the expression of NSC makers and up regulated neuronal markers. In the standard cell culture system, Nestin and SOX2 were expressed even after 14 days of

differentiation. In contrast, in the co-culture conditions significant down regulation was observed within 24hrs.

When the expression of markers were characterized over 7 days, it was observed that the down regulation of Nestin was maintained at the level of 20% of total cells, from 24 hrs through a duration of the 7 days in co-culture. Nestin is an accepted NSC marker (Gilyarov 2008; Lendahl, et al. 1990); it is required for NSC self-renewal (Park, et al. 2010) and is down regulated in differentiating cells by a mechanism involving the proteasome and Notch signaling (Mellodew, et al. 2004). Notch signaling is responsible for lateral inhibition of differentiation, in populations of NSCs that interact by cell-cell contact (Mellodew, et al. 2004). As in the *in-vivo* NSC niche, this lateral inhibition keeps NSCs from differentiating (Lathia, et al. 2008; Mellodew, et al. 2004). As such, this could be an explanation for the continuous up-regulation of Nestin in 'pure' NSC cultures and their low yield of differentiation. In the standard culture condition, NSCs are in continuous contact with each other and thus lateral inhibition via Notch signaling between NSCs could inhibit the differentiation of neighboring cells, restricting or hindering their differentiation (Ferrón, et al. 2011; Mellodew, et al. 2004). In the co-culture condition, however, NSCs are exposed to cells of different lineages and probably lower levels of Notch / delta expression. Upon contact with the feeder cells lateral inhibition via Notch signaling probably did not occur and Nestin was targeted to the proteasome and degraded from the cell (Mellodew, et al. 2004); this could possibly also be one of the factors that contributed toward accelerated and enhanced differentiation of NSCs in the co-culture system. It is also in line with the fact that NSCs migrate out of the NSC niche as they differentiate into different neuronal lineages (Demir, et al. 2009; Watt

and Hogan 2000). As NSCs migrate out of the stem cell niche, they are no longer in contact with the niche NSCs and they thus differentiate.

Ki 67 expression was also down-regulated rapidly as its expression became negligible by the 2$^{nd}$ day of co-culture. As Nestin plays a role in self-renewal, it could be responsible in the rapid down regulation of Ki 67 (Park, et al. 2010). It could be argued that the absence of mitogenic factors could have triggered the down-regulation of Ki 67. However, in the standard culture condition, Ki 67 was retained till 4 days after the removal of mitogenic factors. Thus, the co-culture condition could trigger an accelerated exit from the cell cycle.

β-III Tubulin, a marker for immature neurons was up-regulated to about 50% of the total GFP cell population at day 5 of co-culture, after which it declined. This could indicate that there is a peak of differentiation towards the neuronal lineage at 5 days of co-culture. Possibly after 5 days, when the cells became more mature, they may down-regulate the expression of intermediate neuronal markers. Alternatively, the *in-vitro* cell culture system was not sufficient to support more mature neurons, which eventually died off as a consequence. As the co-cultured NSCs rapidly exited the cell cycle, there is also a shortage of co-cultured cells to maintain neuronal cell numbers.

Upon studying a more mature neuronal phenotypic marker for neurons, TH, my data showed that this co-culture system did not significantly enhance the differentiation of DA neurons; additional cues seem to be necessary for achieving DA differentiation. However, due to the generally low TH yield, it is possible that a larger sample size is needed before any conclusion can be made (Moher, et al. 1994).

As in the standard culture method, co-cultured cells were not electrophysiologically apt as they did not elicit action potentials. This could indicate that more than 24hrs of co-culture would be necessary before action potential could be formed. However, the method used to isolate these cells for electrophysiological analysis was rigorous and possibly damaging to cells. The co-cultured cells were trypsinized and strained with a strainer of pore size 70μm (BD, Franklin Lakes, NJ), so as to ensure that cells were not attached together. GFP positive cells were then FACS sorted and re-seeded onto laminin coated cover-slips for ~ 24hrs before analysis. During the course of this procedure, mechanical and physical stresses the cells were subjected to could have modified and even eliminated the cells of interest (Zaitoun, et al. 2010; Avvisato, et al. 2007; Alenghat and Ingber 2002). Mature neuronal cells tend to be more vulnerable due to their highly polarized and intricate morphology with extended neurite outgrowths and complex neurite branchings. Furthermore, the entire procedure of trypsinization and sorting takes many hours, which might harm or kill cells. In addition, GFP cells were probed only 24 hrs after being sorted out of the co-culture population. Thus, they might not be representative of the cells observed in the direct co-culture assay and it is possible that such cells could de-differentiate when removed from their differentiating environment (Gritti Angela, et al. 2001; Clarke, et al. 2000). Therefore, a procedure that allows cells to be probed electrophysiologically while within the co-culture environment should be utilized before a sound conclusion on the potential of electrophysiological maturation in the co-culture conditions can be made.

To summarize, the co-culture condition induced NSCs to rapidly (within 12 hrs) exhibit neuronal morphology with elongated neurite out-growths and complex neurite

branchings. It also rapidly down regulated NSC markers and up regulated neuronal markers. Differentiation via the co-culture condition was also enhanced qualitatively because the final percentage of cells without the expression of NSC markers and with the expression of neuronal markers is significantly higher than seen on cells differentiated under standard culture conditions.

**Direct cell-cell contact between living cells is required for accelerated and enhanced differentiation**

The effect of accelerated and enhanced differentiation was not induced when NSCs were co-cultured on a layer of undifferentiated NSCs or in other words, the underlying layer of cells need to be pre-differentiated for ~3 days so as to be able to induce this effect. This means that the differentiated status of the underlying feeder layer of cells was very crucial to the effect. This also eliminated the possibility that an increase in total cell density could have caused this effect. In the co-culture condition, the total cellular density, which has been shown to be a factor in inducing neuronal differentiation and maturation of stem cells (Wang and Kisaalita 2011; El-Akabawy, et al. 2011) is increased as NSCs were cultured with the underlying layer of confluent cells. But, as NSCs did not undergo enhanced differentiation when co-cultured on undifferentiated feeder cells of similar cell numbers, this ruled out cell density as the major triggering element.

Vazin and colleagues (2008) showed that human embryonic stem cells, when co-cultured on fixed PA6 feeder cells induced the same number of neuronal cells as when co-cultured on live PA6 cells, therefore indicating a role for either physical / topographical cues or by membrane – anchored molecules / surface molecules in

inducing neuronal differentiation (Vazin, et al. 2008) (Kawasaki, et al. 2000). My data shows that NSCs co-cultured onto a layer of fixed neural cells were not induced to undergo accelerated and enhanced differentiation, indicating that the underlying feeder layer of cells needed to be living cells. It also indicated that physical / topographical cues were probably not involved in evoking accelerated and enhanced differentiation.

Midbrain specific astrocytes have been shown to stimulate DA neuron generation at a markedly higher density than astrocytes of other brain regions, thus indicating a region specific effect (Li, et al. 2009). In addition, Vorasubin and colleagues (2007) showed that NSC co-cultured on grafts of different brain regions assumed the differentiation phenotype of those respective regions (Vorasubin, et al. 2007). These studies indicated region specific influence to NSC differentiation. In the same way, to understand if accelerated and enhanced differentiation was specific to the mid-brain origin of the feeder layer or specific to the cell-line, NSCs derived from the fetal cortex were used as feeder layers. Enhanced differentiation was seen on both feeder types, thus indicating that this phenomenon is not specific to the particular cell line, but could possibly be a neuronal phenomenon. However, it should be noted that the co-cultured GFP-NSCs used in all these experiments were identical – GFP-NSCs derived from the ventral mesencephalon of a 10-week old fetus. Thus, to better understand if this phenomenon was region specific or if it was a neuronal phenomenon, co-cultured NSCs from different brain regions, including those of primary fetal source should be tested.

I moved on to hypothesize that either soluble factors, the ECM or direct cell contact could be involved in the accelerated and enhanced neuronal differentiation. In a first set of experiments I ruled out that soluble factors contributed to this effect.

Conditioned media (CM) collected from differentiating NSC populations were unable to induce accelerated and enhanced differentiation. CM is commonly used to study the effect of cytokines, growth factors and other soluble molecules on other cells and numerous studies have shown that NSCs are influenced by soluble factors. For example, CM from MSCs has been shown to increase the viability of mature neurons and the distinct glial cell populations, indicating a role of secreted diffusible factors (Ribeiro, et al. 2011). It has also been shown that the secreted factors of ramified microglial cells have a role in astrogliogenesis and in the maintenance of NSCs via the Stat3 function (Zhu, et al. 2008). Furthermore, the CM from PA6 cells has been shown to induce hESCs to differentiate into dopaminergic neurons (Kawasaki, et al. 2000; Vazin, et al. 2008). However, in the context of accelerated and enhanced differentiation, CM failed to drive NSCs into rapid differentiation when they were cultured with CM collected from a layer of predifferentiated feeder cells.

To eliminate the argument that the candidate factors were not concentrated enough in the CM, the CM was concentrated and NSCs were seeded in these concentrated media. Again, the results from these set of experiments supported that conclusion that secreted and diffusible factors were not key players in triggering enhanced differentiation.

Paracrine and feeder layer triggered autocrine signals may have a very short half-life (d'Angelo, et al. 2010) and may only trigger cells in close proximity (Tang, et al. 2010). As such, if such signals were involved, they cannot be studied via the CM approach. Based on the assumption that any soluble factors would act in a dose dependent manner on the GFP-NSCs, the volume of the cell culture medium was

increased in order to dilute any potential factors below threshold levels. If soluble factors were involved, differences in the concentration of the factors by changing media volumes should influence the effect of enhanced differentiation. However, any dependency on the volume of the culture medium was not observed. Also, differences in accelerated and enhanced differentiation were not observed when culture media was continually changed in order to remove the possibility of any accumulation of soluble factors in the medium. Together, the data strongly suggests that diffusible factors released into the medium are not the key component of accelerated and enhanced differentiation.

In order to study the role of ECM, the predifferentiated feeder layer cells were decellularized by the use of 2 established methods (Peng, et al. 2011) which removes any cellular component but leaves behind the ECM deposited by cells. When undifferentiated NSCs were seeded on top of ECM derived from predifferentiated NSCs I did not observe any accelerated or enhanced differentiation. The expression of Nestin, β-tubulin III as well as neurite outgrowth and number of branches were unaffected when NSCs were grown on EMC. This conclusion was supported by results from experiments in which ECM deposition was enhanced using the method of macromolecular crowding (MMCs). MMCs produces an “excluded volume effect” which has been shown to create a more physiological cell culture environment and to enhance the ECM deposition of cells (Chen, et al. 2011; Ellis 2001). As in the experiments with non-enhanced ECM, MMC did not result in accelerated and enhanced differentiation of NSCs grown on MMC derived ECM. Taken together, I concluded that the ECM does not induce the accelerated and enhanced differentiation of NSCs.

Having ruled out soluble factors and the ECM as being inducers of enhanced differentiation, I moved on to study direct cell-cell interactions as a possible mechanism. I hypothesized that direct cell junctions were involved in rapid differentiation and I tested this experimentally. Of the 3 general types of cell junctions (tight junctions, gap junctions and anchoring junctions), tight junctions are generally predominant in epithelial cells and not in neural cells (Lodish 2008). Furthermore, tight junctions function primarily as occluding junctions in vertebrates that create a barrier or a semi-permeable membrane between cells (Alberts 2008). Gap junctions and anchoring junctions, on the other hand, have roles in cell-cell signaling (Alberts 2008; Lodish 2008) and are known to be involved in neural development, differentiation and function (de Rivero Vaccari, et al. 2007; Lee and Reichardt; Kunze, et al. 2009; Elias and Kriegstein 2008; Zhang 2010; Todd, et al. 2010; Bani-Yaghoub, et al. 1999; Elias, et al. 2007; Jaderstad, et al. 2010; Ranscht 1991; Wiertz, et al. 2011; Steinberg and McNutt 1999). In the developing nervous system, neurons are coupled by gap junctions (de Rivero Vaccari, et al. 2007) and they play important roles in neurogenesis (Becker and Mobbs 1999), neuronal differentiation (Bani-Yaghoub, et al. 1999), neuroblast migration (Lo Turco and Kriegstein 1991), synaptogenesis (de Rivero Vaccari, et al. 2007), and neural circuit formation (Kandler and Katz 1995; Peinado, et al. 1993). Similarly, cadherin mediated anchoring junctions are involved in maintaining the in-vivo NSC niche (Zhang, et al. 2010) and in neuronal development (Ranscht 1991); thus, I focused my approach on gap junctions and anchoring junctions. To study the effect of gap junctions, GFP-NSCs were seeded onto predifferentiated feeder cells with or without the inhibitors and in the control

condition with or without the inhibitors. After 24hrs of culture, co-cultured GFP cells were assayed for extensions in neurite length and the branching complexity.

Interestingly, the effect of accelerated differentiation was blocked when inhibitors of gap junctions were included in the cell culture medium, thus suggesting that gap junctions could play a major role in accelerated differentiation. Though the number of cells attached to the feeder was reduced under the influence of gap junction blockers, neurite outgrowth could not be observed whereas the co-cultured cells without the blocker show enhanced differentiation. However, the control cells (NSCs + inhibitor without co-culture) and the co-cultured cells with the inhibitor (NSCs + inhibitor in co-culture) had a similar morphology, which seems to be abnormal. Ideally, cells in the control condition with or without the inhibitor should posses an identical morphology. Unfortunately, this was not observed. It could be argued that the gap junction blocker disrupted the adhesion property of gap junctions, and thus caused GFP cells to be lower in number and to possess truncated morphologies. It could be suggested that the inhibition of accelerated differentiation was due to a toxicity effect or a secondary neural effect due to the inhibition of gap junctions. In support with the latter, it has been shown that gap junctions are required for any kind of neural differentiation and that gap junction inhibition blocks differentiation altogether (Bani-Yaghoub, et al. 1999). Thus, the results we get may be due to an inhibition of differentiation, which naturally would also inhibit accelerated and enhanced differentiation of co-cultured NSCs.

In a next step, I explored whether anchoring junctions were involved in accelerated and enhanced differentiation. Anchoring junctions are composed of desmosomes, hemidesmosomes and adherens junctions (Alberts 2008; Lodish 2008).

Hemidesmosomes functions to adhere cells to the ECM (Lodish 2008). As the role of the ECM had been eliminated from previous experiments, the role of hemidesmosomes were not considered. Desmosomes and adherens junctions function in direct cell-cell interactions and both of these are mediated by cadherins (Zhang, et al. 2010; Meyer, et al. 1992; Lodish 2008). There are more than 80 members of cadherins which are grouped into at least 6 sub-families (Alberts 2008; Steinberg and McNutt 1999; Ranscht 1991; Yagi and Takeichi 2000). Of these cadherins, N-Cadherins are specifically predominant in the nervous system (Peng and Raghunath 2010; Ranscht 1991). Thus, in studying the role of anchoring junctions, I chose to inhibit N-CADs by using a specific blocking antibody against this major cadherin in the brain (Wiertz, et al. 2011). Results indicated that the effect of accelerated differentiation was reduced in a dose dependent manner. Unlike gap junction blockers, the inhibition of N-CADs did not disrupt the morphology of cells that were not in the co-culture condition, but were cultured with the inhibitor; cells in the control condition (NSCs + inhibitor without co-culture) had a similar morphology to the cells that had no underlying feeder layer of predifferentiated cells and were cultured without the inhibitor. Thus, this indicates that N-CADs may play a pivotal role in the rapid differentiation of co-cultured NSCs.

Various studies have shown that Cadherins work in conjunction with gap-junctions during cellular interaction (Govindarajan, et al. 2010; Guo, et al. 2003; Meyer, et al. 1992; Fujimoto, et al. 1997); Cadherins, including N-Cadherins are used to hold cells together so as to achieve the assembly of gap junction components (Fujimoto, et al. 1997); thus, it is possible that both these junctions are formed in unison. Therefore, my data could suggest that accelerated and enhanced differentiation is mediated by a cell

contact related mechanism involving gap and anchoring junctions. However, further work needs to be done so as to understand the details as to how each of these junctions are involved.

While gap junctions have been shown to have varied functions, it should be noted that N-CADs have tend to be more involved in the proliferation and in the maintanence of stemness within NSCs (Zhang et al. 2010), thus contradicting my data. However, though in minority, other studies support my results. N-CADs have been shown to be involved in neurite outgrowth (Bixby et al. 1990), axonal migration and neuronal development (Doherty et al. 1991).

To support this model further, in the future gap junctions and N-CADs could be knocked-down genetically by the siRNA approach (Jaderstad, et al. 2010). Furthermore, more than 5 different gap junctions involving different connexin proteins are necessary for different neural processes (Dermietzel and Spray 1993). The inhibition of gap junctions in my experiments unselectively blocked all types of gap junctions. Utilizing siRNA approaches, specific connexin proteins could be disrupted so as to understand which particular gap junction might be the mediator of accelerated and enhanced differentiation.

Furthermore, my experiments did not study the role for other types of CAMs, which could be involved in the enhanced and accelerated differentiation of NSCs. In light of having a more mechanistic understanding of this phenomenon, NSC interactions mediated by other types of CAMS should be studied.

**Generation of a DDC-GFP reporter NSC line**

Having created and characterized a system for accelerated and enhanced differentiation of NSCs, I decided to put this system to a further test. I had generated a DDC-GFP promoter-reporter cell line and found that within the cell population, there were two distinct populations of cells; one population showed a basal level of GFP expression while the other population of cells did not. Using my novel cell culture method, I showed that the population of cells that did not express basal levels of GFP expression was most feasible to be used as a promoter-reporter cell line, that faithfully reflects the expression of the native DDC protein. Unlike the standard culture system that required 7-14 days of culture (Li, et al. 2009) for achieving neuronal differentiation, the co-culture system produced differentiated cells within 24hrs. However, it could be argued that this system required a predifferentiated layer of cells, which elongates the total duration of culture. Based on the results in chapter 5, a minimum of ~ 3 days of predifferentiation is sufficient to induce accelerated differentiation. Thus, with the co-culture system, it is possible that the total culture time amounts to only 4 days while the standard culture conditions requires a minimum of about 7 but up to 21 days (Gritti Angela, et al. 2001) of differentiation. Therefore, the co-culture system brings a major benefit of time for neuronal differentiation.

For prospective clinical utilization of these enhanced neural cells, a better understanding of the underlying mechanisms that drive accelerated and enhanced differentiation is required. With a better understanding of the mechanism, it may be possible to reverse-engineer the process by introducing the active components that trigger enhanced differentiation into the cell culture flask, enabling NSCs to undergo accelerated

differentiation without the presence of the underlying pre-differentiated layer of cells. At present, without this mechanistic understanding, the presence of the feeder cells is a limitation to any clinical application of rapidly differentiating cells. Though the feeder layer is robust in accelerating and enhancing NSC differentiation, the separation of the NSCs from the feeder layer is difficult. Further, the feeder cells may evoke physiologically unfavorable effects, which are yet to be studied.

Apart from the feeder layer, the use of cell lines is another limitation. As NSC lines have been genetically manipulated and immortalized, they may have functional artifacts not present in the *in-vivo* system. Though it was shown that this phenomenon is probably not a cell culture artifact or specific to a particular cell line, studies should be repeated with primary human NSCs so as to further rule out the possibility that accelerated and enhanced differentiation could be an artifact.

There were significantly lesser GFP cells in the co-culture condition, as compared to the cells in the control condition. Though the explanation for this was not directly studied, it could be speculated as follows: It was shown that GFP cells in the co-cultured condition accelerated their exit from the cell cycle. As such, while cells in the control coindition undergo cell-division thus increasing their numbers, cells in the co-culture condition either differentiate or undergo cell death. Furthermore, the co-culture condition may evoke selection criteria, which induces some cells to differentiate while pushing other cells toward cell death. Thus, these reasons could possibly be the reason for the significantly lower cell numbers.

Though this study predominantly investigated and aimed at neuronal differentiation, fully functional, electorphysiologically mature NSCs were not found.

Numerous studies have shown that signaling molecules such as SHH and FFG8 (Enver, et al. 2009) enhanced DA differentiation and maturation. Furthermore, studies have shown that ECM components (Cho, et al. 2008; Silva, et al. 2009; Blow 2009; Meyer, et al. 2009), biophysical signaling (Wang and Spector 2009), mechanical stimuli and nanotopographical cues play a role in NSC differentiation. Understanding the mechanisms governing these unique differentiation protocols together with the mechanism governing the differentiation of NSCs via the co-culture method could lead to the development of *in-vitro* cell culture systems that would allow us to generate neurons with a specific neurotransmitter phenotype efficiently and in high purity within a short time frame that could possibly be of clinical relevance to ailments of the nervous system.

**Summary**

This study validated the hypothesis that a cell culture environment that allows cells to be in communication with cells of different lineages and different stages of differentiation, interacting with dynamic extrinsic cues, as in the *in-vivo* environment, would be more efficient and effective in supporting neuronal differentiation. The major achievement of this work is that a co-culture system has been devised which results in accelerated and enhanced neuronal differentiation as compared to the existing cell culture methods used. I have characterized this system in greater detail and I also have shown that gap junctions and anchoring junctions may play a major role in the accelerated and enhanced differentiation process. It would be worthwhile to continue to study the exact cellular and molecular mechanisms underlying this process. Upon understanding the cellular, molecular and biophysical mechanisms in more detail, a cell culture system could possibly be engineered which would remove the presence of the underlying layer

of cells and substitute cells by the controlled addition of appropriate factors to the cell culture system. Also, the underlying feeder layer might contain a wide spectrum of signals, which may also may include inhibitory molecules that restrict the differentiation of NSCs (Gritti Angela, et al. 2001; Enver, et al. 2009); removing the unnecessary ‘noise’ by reverse engineering the essential components driving differentiation could thus provide an efficient system of neuronal differentiation possibly facilitating to generate neurons with high efficiency and purity with future clinical relevance.

# Bibliography

**References**


Abrams, G A, S L Goodman, P F Nealey, M Franco, and C J Murphy. "Nanoscale topography of the basement membrane underlying the corneal epithelium of the rhesus macaque." *Cell Tissue Res* 299, no. 1 (2000): 39-46.

Adelman, George, and Barry H Smith. "Stem Cells and CNS Repair." In *Encyclopedia of neuroscience*2213. Elsevier Science Ltd, 1999.

Alberts, Bruce. *Molecular biology of the cell.* Other, 2008.

Alenghat, Francis J, and Donald E Ingber. "Mechanotransduction: all signals point to cytoskeleton, matrix, and integrins." *Sci STKE* 2002, no. 119 (2002): pe6.

Altmann, C R, and A H Brivanlou. "Neural patterning in the vertebrate embryo." *Int Rev Cytol* 203 (2001): 447-82.

Avvisato, Christopher L, Xiang Yang, Salim Shah, Becky Hoxter, Weiqun Li, Richard Gaynor, Richard Pestell, Aydin Tozeren, and Stephen W Byers. "Mechanical force modulates global gene expression and beta-catenin signaling in colon cancer cells." *J Cell Sci* 120, no. Pt 15 (2007): 2672-82.

Baharvand, Hossein. "Mechanotransduction and Its Role in Stem Cell Biology." In *Trends in stem cell biology and technology*413. Humana Pr Inc, 2009.

Bani-Yaghoub, M., T.M. Underhill, and C.C.G. Naus. "Gap junction blockage interferes with neuronal and astroglial differentiation of mouse P19 embryonal carcinoma cells." *Dev Genet* 24, no. 1-2 (1999): 69-81.

Barakat, I, M Sensenbrenner, and G Vincendon. "The importance of cell contact for the proliferation of neuroblasts in culture and its stimulation by meningeal extract." *Neurochem Res* 7, no. 3 (1982): 287-300.

Bean, BP. "The action potential in mammalian central neurons." *Nature Reviews Neuroscience* 8, no. 6 (2007): 451-51.

Bear, Mark F, Barry W Connors, and Michael A Paradiso. “Neuroscience”. 2007.

Becker, DL, and P Mobbs. "Connexin alpha1 and cell proliferation in the developing chick retina." *Exp Neurol* (1999): 326-32.

Benjamin, I J. "Matrix metalloproteinases: from biology to therapeutic strategies in cardiovascular disease." *J Investig Med* 49, no. 5 (2001): 381-97.

Bernal, Giovanna M, and Daniel A Peterson. "Phenotypic and gene expression modification with normal brain aging in GFAP-positive astrocytes and neural stem cells." *Aging Cell* 10, no. 3 (2011): 466-82.

Bito, H, K Deisseroth, and R W Tsien. "Ca2+-dependent regulation in neuronal gene expression." *Curr Opin Neurobiol* 7, no. 3 (1997): 419-29.

Bixby JL and Zhang R. "Purified N-cadherin is a potent substrate for the rapid induction of neurite outgrowth." Journal of Cell Biology (1990) vol. 110 no. 4 1253-1260

Blow, N.. "Cell culture: building a better matrix." *Nat Methods* 6, no. 8 (2009): 619-22.

Brazel, Christine Y, Tobi L Limke, Jihan K Osborne, Takumi Miura, Jingli Cai, Larysa Pevny, and Mahendra S Rao. "Sox2 expression defines a heterogeneous population of neurosphere-forming cells in the adult murine brain." *Aging Cell* 4, no. 4 (2005): 197-207.

Campos, Lia S, Dino P Leone, Joao B Relvas, Cord Brakebusch, Reinhard Fässler, Ueli Suter, and Charles ffrench-Constant. "Beta1 integrins activate a MAPK signalling pathway in neural stem cells that contributes to their maintenance." *Development* 131, no. 14 (2004): 3433-44.

Campos, Lia Scotti. "Beta1 integrins and neural stem cells: making sense of the extracellular environment." *Bioessays* 27, no. 7 (2005): 698-707.

Carr, Chavela M, and Mary Munson. "Tag team action at the synapse." *EMBO Rep* 8, no. 9 (2007): 834-38.

Chao, Daniel L, Le Ma, and Kang Shen. "Transient cell-cell interactions in neural circuit formation." *Nat Rev Neurosci* 10, no. 4 (2009): 262-71.

Chatelin, S.. "Neuronal promoter of human aromatic L-amino acid decarboxylase gene directs transgene expression to the adult floor plate and aminergic nuclei induced by the isthmus." *Brain Res Mol Brain Res* 97, no. 2 (2001): 149-60.

Chen, Clarice, Felicia Loe, Anna Blocki, Yanxian Peng, and Michael Raghunath. "Applying macromolecular crowding to enhance extracellular matrix deposition and its remodeling in vitro for tissue engineering and cell-based therapies." *Adv Drug Deliv Rev* 63, no. 4-5 (2011): 277-90.

Cheng, Ke, and William S Kisaalita. "Exploring cellular adhesion and differentiation in a micro-/nano-hybrid polymer scaffold." *Biotechnol Prog* 26, no. 3 (2010): 838-46.

Cho, Myung Soo, Young-Eun Lee, Ji Young Kim, Seungsoo Chung, Yoon Hee Cho, Dae-Sung Kim, Sang-Moon Kang, Haksup Lee, Myung-Hwa Kim, Jeong-Hoon Kim, Joong Woo Leem, Sun Kyung Oh, Young Min Choi, Dong-Youn Hwang, Jin Woo

Chang, and Dong-Wook Kim. "Highly efficient and large-scale generation of functional dopamine neurons from human embryonic stem cells." *Proc Natl Acad Sci U S A* 105, no. 9 (2008): 3392-97.

Clarke, D L, C B Johansson, J Wilbertz, B Veress, E Nilsson, H Karlström, U Lendahl, and J Frisén. "Generalized potential of adult neural stem cells." *Science* 288, no. 5471 (2000): 1660-63.

Conti, Luciano, and Elena Cattaneo. "Neural stem cell systems: physiological players or in vitro entities?" *Nat Rev Neurosci* 11, no. 3 (2010): 176-87.

Da Silva, JS, and CG Dotti. "Breaking the neuronal sphere: regulation of the actin cytoskeleton in neuritogenesis." *Nature Reviews Neuroscience* 3, no. 9 (2002): 694-94.

d'Angelo, Ivana, Marcos Garcia-Fuentes, Yolanda Parajó, Alexander Welle, Tibor Vántus, Anikó Horváth, Gyorgyi Bokonyi, Gyorgy Kéri, and Maria José Alonso. "Nanoparticles Based on PLGA:Poloxamer Blends for the Delivery of Proangiogenic Growth Factors." *Mol Pharm* (2010)

D'Ascenzo, Marcello, Roberto Piacentini, Patrizia Casalbore, Manuela Budoni, Roberto Pallini, Gian Battista Azzena, and Claudio Grassi. "Role of L-type Ca2+ channels in neural stem/progenitor cell differentiation." *Eur J Neurosci* 23, no. 4 (2006): 935-44.
de Rivero Vaccari, J. C., R. A. Corriveau, and A. B. Belousov. "Gap junctions are required for NMDA receptor dependent cell death in developing neurons." *J Neurophysiol* 98, no. 5 (2007): 2878-86.

Demir, Ozlem, Sheila Singh, Lars Klimaschewski, and Isil Aksan Kurnaz. "From birth till death: neurogenesis, cell cycle, and neurodegeneration." *Anat Rec (Hoboken)* 292, no. 12 (2009): 1953-61.

Dermietzel, R., and D.C. Spray. "Gap junctions in the brain: where, what type, how many and why?" *Trends Neurosci* 16, no. 5 (1993): 186-92.

Ding, Sheng, and Peter G Schultz. "A role for chemistry in stem cell biology." *Nat Biotechnol* 22, no. 7 (2004): 833-40.

Ditlevsen, Dorte Kornerup, and Kateryna Kolkova. "Signaling Pathways Involved in NCAM-Induced Neurite Outgrowth." *Neurochem Res* (2008)

Ditlevsen, Dorte Kornerup, Gro Klitgaard Povlsen, Vladimir Berezin, and Elisabeth Bock. "NCAM-induced intracellular signaling revisited." *J Neurosci Res* 86, no. 4 (2008): 727-43.

Doherty P and Walsh FS. " The contrasting roles of N-CAM and N-Cadherins as neurite outgrowth-promoting molecules."Journal of Cell Science (1991) Vol 15: 13-21

Donato, Roberta, Erik A Miljan, Susan J Hines, Sihem Aouabdi, Kenneth Pollock, Sara Patel, Frances A Edwards, and John D Sinden. "Differential development of neuronal physiological responsiveness in two human neural stem cell lines (1)." *BMC Neurosci* 8, no. 1 (2007): 36-36.

Du, Jing, Xiaofei Chen, Xudong Liang, Guangyao Zhang, Jia Xu, Linrong He, Qingyuan Zhan, Xi-Qiao Feng, Shu Chien, and Chun Yang. "Integrin activation and internalization on soft ECM as a mechanism of induction of stem cell differentiation by ECM elasticity." *Proc Natl Acad Sci U S A* 108, no. 23 (2011): 9466-71.

El-Akabawy, Gehan, Lourdes Martinez Medina, Aaron Jeffries, Jack Price, and Michel Modo. "Purmorphamine Increases DARPP-32 Differentiation in Human Striatal Neural Stem Cells Through the Hedgehog Pathway." *Stem Cells Dev* (2011)

Elias, L, and A Kriegstein. "Gap junctions: multifaceted regulators of embryonic cortical development." *Trends Neurosci* 31, no. 5 (2008): 243-50.

Elias, Laura A. B., Doris D. Wang, and Arnold R. Kriegstein. "Gap junction adhesion is necessary for radial migration in the neocortex." *Nature* 448, no. 7156 (2007): 901-07.

Elias, Laura A. B., Doris D. Wang, and Arnold R. Kriegstein. "Gap junction adhesion is necessary for radial migration in the neocortex. (1)." *Nature* 448, no. 7156 (2007): 901-07.

Ellis, R.J.. "Macromolecular crowding: an important but neglected aspect of the intracellular environment." *Curr Opin Struct Biol* 11, no. 1 (2001): 114-19.

Emoto, Kazuo. "[Impacts of ECM dynamics on neuronal remodeling]." *Seikagaku* 82, no. 10 (2010): 972-78.

Enver, Tariq, Martin Pera, Carsten Peterson, and Peter W Andrews. "Stem cell states, fates, and the rules of attraction." *Cell stem cell* 4, no. 5 (2009): 387-97.

Erlander, M G, N J Tillakaratne, S Feldblum, N Patel, and A J Tobin. "Two genes encode distinct glutamate decarboxylases." *Neuron* 7, no. 1 (1991): 91-100.

Even-Ram, Sharona, Vira Artym, and Kenneth M Yamada. "Matrix control of stem cell fate." *Cell* 126, no. 4 (2006): 645-47.

Ferrón, Sacri R, Marika Charalambous, Elizabeth Radford, Kirsten McEwen, Hendrik Wildner, Eleanor Hind, Jose Manuel Morante-Redolat, Jorge Laborda, Francois Guillemot, Steven R Bauer, Isabel Fariñas, and Anne C Ferguson-Smith. "Postnatal loss of Dlk1 imprinting in stem cells and niche astrocytes regulates neurogenesis." *Nature* 475, no. 7356 (2011): 381-85.

Fetcho, J R, and D M O'Malley. "Imaging neuronal networks in behaving animals." *Curr Opin Neurobiol* 7, no. 6 (1997): 832-38.

Friling, Stina, Elisabet Andersson, Lachlan H Thompson, Marie E Jönsson, Josephine B Hebsgaard, Evanthia Nanou, Zhanna Alekseenko, Ulrika Marklund, Susanna Kjellander, Nikolaos Volakakis, Outi Hovatta, Abdeljabbar El Manira, Anders Björklund, Thomas Perlmann, and Johan Ericson. "Efficient production of mesencephalic dopamine neurons by Lmx1a expression in embryonic stem cells." *Proc Natl Acad Sci U S A* 106, no. 18 (2009): 7613-18.

Fujimoto, K, A Nagafuchi, S Tsukita, A Kuraoka, A Ohokuma, and Y Shibata. "Dynamics of connexins, E-cadherin and alpha-catenin on cell membranes during gap junction formation." *J Cell Sci* 110, no. 3 (1997): 311.

Gage, Fred H. "Neurogenesis in the adult brain." *J Neurosci* 22, no. 3 (2002): 612-13.

Gallagher, H C, O A Odumeru, and C M Regan. "Regulation of neural cell adhesion molecule polysialylation state by cell-cell contact and protein kinase C delta." *J Neurosci Res* 61, no. 6 (2000): 636-45.

Gao, W O, N Heintz, and M E Hatten. "Cerebellar granule cell neurogenesis is regulated by cell-cell interactions in vitro." *Neuron* 6, no. 5 (1991): 705-15.

Gilyarov, A V. "Nestin in central nervous system cells." *Neurosci Behav Physiol* 38, no. 2 (2008): 165-69.

Glaser, Tamara, Steven M Pollard, Austin Smith, and Oliver Brüstle. "Tripotential differentiation of adherently expandable neural stem (NS) cells." *PloS one* 2, no. 3 (2007): e298.

Goldman, S A. "Adult neurogenesis: from canaries to the clinic." *J Neurobiol* 36, no. 2 (1998): 267-86.

Goldman, Steve. "Stem and progenitor cell-based therapy of the human central nervous system." *Nat Biotechnol* 23, no. 7 (2005): 862-71.

Götz, Magdalena. "Glial cells generate neurons--master control within CNS regions: developmental perspectives on neural stem cells." *Neuroscientist* 9, no. 5 (2003): 379-97.

Götz, Magdalena, Eva Hartfuss, and Paolo Malatesta. "Radial glial cells as neuronal precursors: a new perspective on the correlation of morphology and lineage restriction in the developing cerebral cortex of mice." *Brain Res Bull* 57, no. 6 (2002): 777-88.

Götz, Magdalena, and Dennis Steindler. "To be glial or not-how glial are the precursors of neurons in development and adulthood?" *Glia* 43, no. 1 (2003): 1-3.
Govindarajan, Rajgopal, Souvik Chakraborty, Kristen E Johnson, Matthias M Falk,

Margaret J Wheelock, Keith R Johnson, and Parmender P Mehta. "Assembly of connexin43 into gap junctions is regulated differentially by E-cadherin and N-cadherin in rat liver epithelial cells." *Mol Biol Cell* 21, no. 23 (2010): 4089-107.

Gritti Angela, Galli Rossella, and Vescovi Angelo Luigi. "Cultures of Stem Cells of the Central Nervous System." In *Protocols for neural cell culture*362. Humana Pr Inc, 2001.

Grupp, Larissa, Hartwig Wolburg, and Andreas F Mack. "Astroglial structures in the zebrafish brain." *J Comp Neurol* 518, no. 21 (2010): 4277-87.

Gtz, M, and WB Huttner. "The cell biology of neurogenesis." *Nat Rev Mol Cell Biol* 6, no. 10 (2005): 777-77.

Guo, Yihe, Cara Martinez-Williams, and D Eugene Rannels. "Gap junction-microtubule associations in rat alveolar epithelial cells." *Am J Physiol Lung Cell Mol Physiol* 285, no. 6 (2003): L1213-21.

Hatten, M E. "Neuronal regulation of astroglial morphology and proliferation in vitro." *J Cell Biol* 100, no. 2 (1985): 384-96.

Hatten, M E. "Neuronal inhibition of astroglial cell proliferation is membrane mediated." *J Cell Biol* 104, no. 5 (1987): 1353-60.

Holmberg, Johan, Emil Hansson, Michal Malewicz, Magnus Sandberg, Thomas Perlmann, Urban Lendahl, and Jonas Muhr. "SoxB1 transcription factors and Notch signaling use distinct mechanisms to regulate proneural gene function and neural progenitor differentiation." *Development* 135, no. 10 (2008): 1843-51.

Hynes, M, J A Porter, C Chiang, D Chang, M Tessier-Lavigne, P A Beachy, and A Rosenthal. "Induction of midbrain dopaminergic neurons by Sonic hedgehog." *Neuron* 15, no. 1 (1995): 35-44.

Hynes, M, K Poulsen, M Tessier-Lavigne, and A Rosenthal. "Control of neuronal diversity by the floor plate: contact-mediated induction of midbrain dopaminergic neurons." *Cell* 80, no. 1 (1995): 95-101.
Hynes, M, and A Rosenthal. "Specification of dopaminergic and serotonergic neurons in the vertebrate CNS." *Curr Opin Neurobiol* 9, no. 1 (1999): 26-36.

Jaderstad, J., L. M. Jaderstad, J. Li, S. Chintawar, C. Salto, M. Pandolfo, V. Ourednik, Y. D. Teng, R. L. Sidman, E. Arenas, E. Y. Snyder, and E. Herlenius. "Communication via gap junctions underlies early functional and beneficial interactions between grafted neural stem cells and the host." *Proceedings of the National Academy of Sciences* 107, no. 11 (2010): 5184-89.

Jandial R, Marc D Binder, Nobutaka Hirokawa, and Uwe Windhorst. "stem cells and CNS repair." In *Encyclopedia of neuroscience*4393. 2008.

Jankovic J. "Parkinson's disease: clinical features and diagnosis." *J. Neurol. Neurosurg. Psychiatr* 79, no. 4 (2008): 368-76.

Jelitai, M, M Anderová, A Chvátal, and E Madarász. "Electrophysiological characterization of neural stem/progenitor cells during in vitro differentiation: study with an immortalized neuroectodermal cell line." *J Neurosci Res* 85, no. 8 (2007): 1606-17.

Juliano, RL. "Signal transduction by cell adhesion receptors and the cytoskeleton: functions of integrins, cadherins, selectins, and immunoglobulin-superfamily members." *Annu Rev Pharmacol Toxicol* 42, no. 1 (2002): 283-83.

Kaji, Keisuke, Katherine Norrby, Agnieszka Paca, Maria Mileikovsky, Paria Mohseni, and Knut Woltjen. "Virus-free induction of pluripotency and subsequent excision of reprogramming factors." *Nature* 458, no. 7239 (2009): 771-75.

Kallos, M S, A Sen, and L A Behie. "Large-scale expansion of mammalian neural stem cells: a review." *Med Biol Eng Comput* 41, no. 3 (2003): 271-82.

Kandel, Eric R, James Harris Schwartz, and Thomas M Jessell. *Principles of neural science.* McGraw-Hill/Appleton & Lange, 2000.

Kandler, K, and L C Katz. "Neuronal coupling and uncoupling in the developing nervous system." *Curr Opin Neurobiol* 5, no. 1 (1995): 98-105.

Kang, Min Kook, and Seung Jin Han. "Post-transcriptional and post-translational regulation during mouse oocyte maturation." *BMB reports* 44, no. 3 (2011): 147-57.

Kawasaki, H, K Mizuseki, S Nishikawa, S Kaneko, Y Kuwana, S Nakanishi, S I Nishikawa, and Y Sasai. "Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity." *Neuron* 28, no. 1 (2000): 31-40.

Kirschenbaum, B, M Nedergaard, A Preuss, K Barami, R A Fraser, and S A Goldman. "In vitro neuronal production and differentiation by precursor cells derived from the adult human forebrain." *Cereb Cortex* 4, no. 6 (1994): 576-89.

Kolahi, Kevin S, and Mohammad R K Mofrad. "Mechanotransduction: a major regulator of homeostasis and development." *Wiley interdisciplinary reviews. Systems biology and medicine* 2, no. 6 (2010): 625-39.

Kriegstein, Arnold, and Arturo Alvarez-Buylla. "The Glial Nature of Embryonic and Adult Neural Stem Cells." *Annu Rev Neurosci* 32, no. 1 (2009): 149-84.

Kunze, A., M. R. Congreso, C. Hartmann, A. Wallraff-Beck, K. Huttmann, P. Bedner, R. Requardt, G. Seifert, C. Redecker, K. Willecke, A. Hofmann, A. Pfeifer, M. Theis, and C. Steinhauser. "Connexin expression by radial glia-like cells is required for neurogenesis in the adult dentate gyrus (1)." *Proceedings of the National Academy of Sciences* 106, no. 27 (2009): 11336-41.

Lahti, Laura, Jonna Saarimäki-Vire, Hannu Rita, and Juha Partanen. "FGF signaling gradient maintains symmetrical proliferative divisions of midbrain neuronal progenitors." *Dev Biol* 349, no. 2 (2011): 270-82.

Lam, Chen Sok, Martin März, and Uwe Strähle. "gfap and nestin reporter lines reveal characteristics of neural progenitors in the adult zebrafish brain." *Dev Dyn* 238, no. 2 (2009): 475-86.

Lareu R, Subramhanya K, Peng Y, Benny P, Chen C, Wand Z, Rajagopalan R, and Raghunath M. "Collagen matrix deposition is dramatically enhanced in vitro when crowded with charged macromolecules: the biological relevance of the excluded volume effect." *FEBS Lett* 581, no. 14 (2007): 2709-14

Lareu, Ricky R, Karthik Harve Subramhanya, Yanxian Peng, Paula Benny, Clarice Chen, Zhibo Wang, Raj Rajagopalan, and Michael Raghunath. "Collagen matrix deposition is dramatically enhanced in vitro when crowded with charged macromolecules: the biological relevance of the excluded volume effect." *FEBS Lett* 581, no. 14 (2007): 2709-14.

Lathia, Justin D, Mark P Mattson, and Aiwu Cheng. "Notch: from neural development to neurological disorders." *J Neurochem* 107, no. 6 (2008): 1471-81.

Lee, S.H., and L.F. Reichardt. "Synaptic adhesion molecules." Cadherins and Synapse Organization. Elsevier Ltd (2009)

Lee, J E. "Basic helix-loop-helix genes in neural development." *Curr Opin Neurobiol* 7, no. 1 (1997): 13-20.

Leipzig, Nic D, and Molly S Shoichet. "The effect of substrate stiffness on adult neural stem cell behavior." *Biomaterials* 30, no. 36 (2009): 6867-78.

Lendahl, U, L B Zimmerman, and R D McKay. "CNS stem cells express a new class of intermediate filament protein." *Cell* 60, no. 4 (1990): 585-95.

Li, Kairong, Bing Xue, Yue Wang, Xuan Wang, Haomin Wang, and Xiaomin Wang. "Ventral mesencephalon astrocytes are more efficient than those of other regions in inducing dopaminergic neurons through higher expression level of TGF-beta3." *J Mol Neurosci* 37, no. 3 (2009): 288-300.

Lo Turco, J J, and A R Kriegstein. "Clusters of coupled neuroblasts in embryonic neocortex." *Science* 252, no. 5005 (1991): 563-66.

Lodish, Harvey F. "Integrating Cells Into Tissues." In *Molecular cell biology*1150. 2008.

Lodish, Harvey F. *Molecular cell biology.* 2008.

Marshall, E. "Gene therapy on trial." *Science* 288, no. 5468 (2000): 951.

Mason, Ivor. "Initiation to end point: the multiple roles of fibroblast growth factors in neural development." *Nat Rev Neurosci* 8, no. 8 (2007): 583-96.

McClellan, A D, D McPherson, and M J O'Donovan. "Combined retrograde labeling and calcium imaging in spinal cord and brainstem neurons of the lamprey." *Brain Res* 663, no. 1 (1994): 61-68.

McNamara, Laura E, Rebecca J McMurray, Manus J P Biggs, Fahsai Kantawong, Richard O C Oreffo, and Matthew J Dalby. "Nanotopographical control of stem cell differentiation." *Journal of tissue engineering* 2010 (2010): 120623.

Mellodew, Karen, Ralf Suhr, Dafe A Uwanogho, Iris Reuter, Urban Lendahl, Helen Hodges, and Jack Price. "Nestin expression is lost in a neural stem cell line through a mechanism involving the proteasome and Notch signalling." *Brain Res Dev Brain Res* 151, no. 1-2 (2004): 13-23.

Merkle, Florian T, Anthony D Tramontin, José Manuel García-Verdugo, and Arturo Alvarez-Buylla. "Radial glia give rise to adult neural stem cells in the subventricular zone." *Proc Natl Acad Sci U S A* 101, no. 50 (2004): 17528-32.

Meyer, RA, DW Laird, JP Revel, and RG Johnson. "Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies." *J Cell Biol* 119, no. 1 (1992): 179.

Meyer, Ulrich, Jörg Handschel, Thomas Meyer, Jorg Handschel, and Hans Peter Wiesmann. "Microenvironmental Determinants of Stem Cell Fate." In *Fundamentals of Tissue Engineering and Regenerative Medicine*1049. Springer Verlag, 2009.

Miller, Freda D, and Andrée Gauthier-Fisher. "Home at last: neural stem cell niches defined." *Cell stem cell* 4, no. 6 (2009): 507-10.

Moher, D, C S Dulberg, and G A Wells. "Statistical power, sample size, and their reporting in randomized controlled trials." *JAMA* 272, no. 2 (1994): 122-24.

Morest, D Kent, and Jerry Silver. "Precursors of neurons, neuroglia, and ependymal cells in the CNS: what are they? Where are they from? How do they get where they are going?" *Glia* 43, no. 1 (2003): 6-18.

Morrison, S J, N M Shah, and D J Anderson. "Regulatory mechanisms in stem cell biology." *Cell* 88, no. 3 (1997): 287-98.

Mousa, Shaker A. "Cell adhesion molecules: potential therapeutic & diagnostic implications." *Mol Biotechnol* 38, no. 1 (2008): 33-40.

Nakase, T. "Gap junctions and neurological disorders of the central nervous system." *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1662, no. 1-2 (2004): 149-58.

Okuno, Kazuto, Shigeru Ohta, Hirofumi Kato, Takashi Taga, Kanji Sugita, and Yoshihiro Takeuchi. "Expression of neural stem cell markers in malignant rhabdoid tumor cell lines." *Oncol Rep* 23, no. 2 (2010): 485-92.

Ono, Yuichi, Tomoya Nakatani, Yoshimasa Sakamoto, Eri Mizuhara, Yasuko Minaki, Minoru Kumai, Akiko Hamaguchi, Miyuki Nishimura, Yoko Inoue, Hideki Hayashi, Jun Takahashi, and Toshio Imai. "Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells." *Development* 134, no. 17 (2007): 3213-25.

Panchision, David M, and Ronald D G McKay. "The control of neural stem cells by morphogenic signals." *Curr Opin Genet Dev* 12, no. 4 (2002): 478-87.

Park, Donghyun, Andy Peng Xiang, Frank Fuxiang Mao, Li Zhang, Chun-Guang Di, Xiao-Mei Liu, Yuan Shao, Bao-Feng Ma, Jae-Hyun Lee, Kwon-Soo Ha, Noah Walton, and Bruce T Lahn. "Nestin is required for the proper self-renewal of neural stem cells." *Stem Cells* 28, no. 12 (2010): 2162-71.

Peinado, A, R Yuste, and L C Katz. "Extensive dye coupling between rat neocortical neurons during the period of circuit formation." *Neuron* 10, no. 1 (1993): 103-14.

Peng, T, MT Bocker, J Holm, WS Toh, CS Hughes, GA Lajoie, T Cao, F Lyko, and M Raghunath. "Human Fibroblast Matrices Bioassembled under Macromolecular Crowding Supports Stable Proliferation of Human Embryonic Stem Cells." *Journal of Tissue Engineering and Regenerative Medicine. 2011 (in review)* (2011)

Peng, Y, and M Raghunath. "Learning from Nature: Emulating Macromolecular Crowding to drive ectracellular metrix enhancement for the creation of connective tissue in vitro." In *Tissue engineering*InTech, 2010.

Peters, A. "The formation and structure of myelin sheaths in the central nervous system." *J Biophys Biochem Cytol* 8, no. 2 (1960): 431.

Pevny, Larysa H., and Silvia K. Nicolis. "Sox2 roles in neural stem cells." *Int J Biochem Cell Biol* 42, no. 3 (2010): 421-24.

Piacentini, Roberto, Cristian Ripoli, Daniele Mezzogori, Gian Battista Azzena, and Claudio Grassi. "Extremely low-frequency electromagnetic fields promote in vitro neurogenesis via upregulation of Ca(v)1-channel activity." *J Cell Physiol* 215, no. 1 (2008): 129-39.

Pollard, Steven M, Luciano Conti, Yirui Sun, Donato Goffredo, and Austin Smith. "Adherent neural stem (NS) cells from fetal and adult forebrain." *Cereb Cortex* 16 Suppl 1 (2006): i112-20.

Ranscht, B. "Cadherin cell adhesion molecules in vertebrate neural development." In *Seminars in Neuroscience*285-96. Elsevier Inc, 1991.

Reynolds, B A, and S Weiss. "Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system." *Science* 255, no. 5052 (1992): 1707-10.

Reynolds, B A, and S Weiss. "Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell." *Dev Biol* 175, no. 1 (1996): 1-13.

Reynolds, Brent A, and Rodney L Rietze. "Neural stem cells and neurospheres--re-evaluating the relationship." *Nat Methods* 2, no. 5 (2005): 333-36.

Ribeiro, C A, A J Salgado, J S Fraga, N A Silva, R L Reis, and N Sousa. "The secretome of bone marrow mesenchymal stem cells-conditioned media varies with time and drives a distinct effect on mature neurons and glial cells (primary cultures)." *J Tissue Eng Regen Med* 5, no. 8 (2011): 668-72.

Ross, Sarah E, Michael E Greenberg, and Charles D Stiles. "Basic helix-loop-helix factors in cortical development." *Neuron* 39, no. 1 (2003): 13-25.

Roy, N S, S Wang, L Jiang, J Kang, A Benraiss, C Harrison-Restelli, R A Fraser, W T Couldwell, A Kawaguchi, H Okano, M Nedergaard, and S A Goldman. "In vitro neurogenesis by progenitor cells isolated from the adult human hippocampus." *Nat Med* 6, no. 3 (2000): 271-77.

Sanai, Nader, Anthony D Tramontin, Alfredo Quiñones-Hinojosa, Nicholas M Barbaro, Nalin Gupta, Sandeep Kunwar, Michael T Lawton, Michael W McDermott, Andrew T Parsa, José Manuel-García Verdugo, Mitchel S Berger, and Arturo Alvarez-Buylla. "Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration." *Nature* 427, no. 6976 (2004): 740-44.

Schmid, Ralf S, and E S Anton. "Role of integrins in the development of the cerebral cortex." *Cereb Cortex* 13, no. 3 (2003): 219-24.

Scholzen, T., and J. Gerdes. "The Ki-67 protein: from the known and the unknown." *J Cell Physiol* 182, no. 3 (2000): 311-22.

Schumm, Michael A, Daniel A Castellanos, Beata R Frydel, and Jacqueline Sagen. "Direct cell-cell contact required for neurotrophic effect of chromaffin cells on neural progenitor cells." *Brain Res Dev Brain Res* 146, no. 1-2 (2003): 1-13.

Siegel, George J, and Bernard W Agranoff. *Basic neurochemistry.* 1999.

Silva, Ana, Jorge Pereira, Catarina R Oliveira, João Bettencourt Relvas, and A Cristina Rego. "BDNF and extracellular matrix regulate differentiation of mice neurosphere-derived cells into a GABAergic neuronal phenotype." *J Neurosci Res* 87, no. 9 (2009): 1986-96.

Sims, K B, J E Crandall, K S Kosik, and R S Williams. "Microtubule-associated protein 2 (MAP 2) immunoreactivity in human fetal neocortex." *Brain Res* 449, no. 1-2 (1988): 192-200.

Soen, Y, A Mori, TD Palmer, and PO Brown. "Exploring the regulation of human neural precursor cell differentiation using arrays of signaling microenvironments." *Mol Syst Biol* 2, no. 1 (2006)

Ståhlberg, Anders, Daniel Andersson, Johan Aurelius, Maryam Faiz, Marcela Pekna, Mikael Kubista, and Milos Pekny. "Defining cell populations with single-cell gene expression profiling: correlations and identification of astrocyte subpopulations." *Nucleic Acids Res* 39, no. 4 (2011): e24.

Steinberg, M S, and P M McNutt. "Cadherins and their connections: adhesion junctions have broader functions." *Curr Opin Cell Biol* 11, no. 5 (1999): 554-60.

Takahashi, A, P Camacho, J D Lechleiter, and B Herman. "Measurement of intracellular calcium." *Physiol Rev* 79, no. 4 (1999): 1089-125.

Tang, Jian, Rong Peng, and Jiandong Ding. "The regulation of stem cell differentiation by cell-cell contact on micropatterned material surfaces." *Biomaterials* 31, no. 9 (2010): 2470-76.

Temple, S, and A A Davis. "Isolated rat cortical progenitor cells are maintained in division in vitro by membrane-associated factors." *Development* 120, no. 4 (1994): 999-1008.

Teo, et al. "Nanotopography/Mechanical Induction of Stem-Cell Differentiation." In *Nuclear Mechanics & Genome Regulation*378. Academic Press, 2010.

Todd, K. L., W. B. Kristan, and K. A. French. "Gap junction expression is required for normal chemical synapse formation." *J Neurosci* 30, no. 45 (2010): 15277-85.

Tsai, R Y, and R D McKay. "Cell contact regulates fate choice by cortical stem cells." *J Neurosci* 20, no. 10 (2000): 3725-35.

Vazin, Tandis, Jia Chen, Chun-Ting Lee, Rose Amable, and William J Freed. "Assessment of stromal-derived inducing activity in the generation of dopaminergic neurons from human embryonic stem cells." *Stem Cells* 26, no. 6 (2008): 1517-25.

Vitalis, Tania, Olivier Cases, and John G Parnavelas. "Development of the dopaminergic neurons in the rodent brainstem." *Exp Neurol* 191 Suppl 1 (2005): S104-12.

Vorasubin, Bonnie, John Weedin, Farid Saljooque, Nathan Wilkes, M Eng, and Hoi Sang U. "Selective differentiation of central nervous system-derived stem cells in response to cues from specific regions of the developing brain." *J Neurosurg* 107, no. 1 (2007): 145-54.

Waites, Clarissa L, Ann Marie Craig, and Craig C Garner. "Mechanisms of vertebrate synaptogenesis." *Annu Rev Neurosci* 28 (2005): 251-74.

Wang, Lina, and William S Kisaalita. "Administration of BDNF/ginsenosides combination enhanced synaptic development in human neural stem cells." *J Neurosci Methods* 194, no. 2 (2011): 274-82.

Wang, Tzu-Wei, and Myron Spector. "Development of hyaluronic acid-based scaffolds for brain tissue engineering." *Acta Biomater* 5, no. 7 (2009): 2371-84.

Watt, F M, and B L Hogan. "Out of Eden: stem cells and their niches." *Science* 287, no. 5457 (2000): 1427-30.

Wegner, Michael, and C Claus Stolt. "From stem cells to neurons and glia: a Soxist's view of neural development." *Trends Neurosci* 28, no. 11 (2005): 583-88.

Wiertz, R W F, E Marani, and W L C Rutten. "Neural cell-cell and cell-substrate adhesion through N-cadherin, N-CAM and L1." *J Neural Eng* 8, no. 4 (2011): 046004.

Williams, RW, and K Herrup. "The control of neuron number." *Annu Rev Neurosci* 11, no. 1 (1988): 423-23.

Yagi, T, and M Takeichi. "Cadherin superfamily genes: functions, genomic organization, and neurologic diversity." *Genes Dev* 14, no. 10 (2000): 1169-80.

Yim, Evelyn K F, Stella W Pang, and Kam W Leong. "Synthetic nanostructures inducing differentiation of human mesenchymal stem cells into neuronal lineage." *Exp Cell Res* 313, no. 9 (2007): 1820-29.

Zaitoun, I, C Erickson, K Schell, and M Epstein. "Use of RNAlater in fluorescence-activated cell sorting (FACS) reduces the fluorescence from GFP but not from DsRed." *BMC research notes* 3, no. 1 (2010): 328.

Zhang, C.. "Gap junctions in olfactory neurons modulate olfactory sensitivity." *BMC Neurosci* 11, no. 1 (2010): 108.

Zhang, Jianing, Gregory J Woodhead, Sruthi K Swaminathan, Stephanie R Noles, Erin R McQuinn, Anna J Pisarek, Adam M Stocker, Christopher A Mutch, Nobuo Funatsu, and Anjen Chenn. "Cortical neural precursors inhibit their own differentiation via N-cadherin maintenance of beta-catenin signaling." *Dev Cell* 18, no. 3 (2010): 472-79.

Zhu, Pengxiang, Ryuji Hata, Fang Cao, Feng Gu, Yasushi Hanakawa, Koji Hashimoto, and Masahiro Sakanaka. "Ramified microglial cells promote astrogliogenesis and maintenance of neural stem cells through activation of Stat3 function." *FASEB J* 22, no. 11 (2008): 3866-77.

# APPENDIX

**DDC Promoter Sequence**

GGGGACAAGTTTGTACAAAAAAGCAGGCTGGtaatcgatggaaatcattgctctccatcatgcgtgagttaaaaccattacttctgagtggtc
agtttacattgggctctgtctagaaggtttgccttcttcacctccagtcacatccccatttcacaagtgagcaaacagccagaggaggggaggaggatggcataagatcttgca
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ctgctcagtaaataatgcagagccggcagctctgattggcttcggggaggcagacactctgtctacataaatggcaatcacatcttctgtgcct**cttaactgtcactgtggaga**
**ggagagagagaggacagagagcaagtcactcccggctgcctttttcacctctgacagagcccagacacc**ACAACTTTGTATACAAAAGTTGT
CCCC

attB1 site : GGGGACAAGTTTGTACAAAAAAGCAGGCTGG
attB5r site : ACAACTTTGTATACAAAAGTTGTCCCC

**Forward primers with attB1 - Recombination Site:**
**5’** GGGACAAGTTTGTACAAAAAAGCAGGCTGGtaatcgatggaaatcattgc **3’**

**Reverse Primers with attB5r - Recombination Site + 31bps regulatory sequence:**
**5’** caagtcactcccggctgcctttttcacctctgacagagcccagacaccACAACTTTGTATACAAAAGTTGTCCCC **3’**

Exon 1 - Follows after the intron
Exon 2 - Regulatory Sequence. Added to the end of the exon 2

**Box 1: DDC Promoter Region and primers**

**eGFP Gene from pEGFP-N1**

GGGGACAACTTTGTATACAAAAGTTGGGcgccaccatggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctgg
acggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctgcccgt
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agtaaTACCCAGCTTTCTTGTACAAAGTGGTCCCC

pEGFP-ClonT-Primers with Gateway Recombination site Seq:
Frd: 5'GGGGACAACTTTGTATACAAAAGTTGGGcgccaccatggtgagcaagggcgagga 3'
Rvs: 5' GGGGACCACTTTGTACAAGAAAGCTGGGTAttacttgtacagctcgtcca 3'

**Box 2: eGFP gene and primers**